TIGHT BINDING BOOK

UNIVERSAL
LIBRARY

OU_164994

UNIVERSAL
LIBRARY

OUP—880—5-8-74—10,000.

OSMANIA UNIVERSITY LIBRARY

Call No. 576/So67 Accession No. 34081

Author

Title Nature of Virus multiplication

This book should be returned on or before the date last marked below.

1953

THE NATURE OF VIRUS MULTIPLICATION

SECOND SYMPOSIUM OF THE
SOCIETY FOR GENERAL MICROBIOLOGY
HELD AT OXFORD UNIVERSITY
APRIL 1952

CAMBRIDGE
Published for the Society for General Microbiology
AT THE UNIVERSITY PRESS
1953

PUBLISHED BY
THE SYNDICS OF THE CAMBRIDGE UNIVERSITY PRESS
London Office: Bentley House, N.W.1
American Branch: New York
Agents for Canada, India and Pakistan: Macmillan

Printed in Great Britain at the Church Army Press
Cowley, Oxford, England

CONTRIBUTORS

(*The names of invited contributors are printed in capitals.*)

Albert, Professor A., Australian National University, Department of Medical Chemistry, 183 Euston Road, London, N.W. 1.

Anderson, Dr E. S., Central Enteric Reference Laboratory, Colindale, London, N.W. 7.

Andrewes, Dr C. H., National Institute for Medical Research, The Ridgeway, Mill Hill, London, N.W. 7.

Backus, Dr R. C., Virus Laboratory, University of California, Berkeley, California, U.S.A.

BAUER, Dr D. J., Wellcome Laboratories of Tropical Medicine, 183 Euston Road, London, N.W. 1.

BAWDEN, Mr F. C., Rothamsted Experimental Station, Harpenden, Herts.

BERGOLD, Dr G. H., Laboratory of Insect Pathology, Department of Agriculture, Sault Ste Marie, Ontario, Canada.

BOYD, Brigadier J. S. K., Wellcome Laboratories of Tropical Medicine, 183 Euston Road, London, N.W. 1.

Brooksby, Dr J. B., Research Station, Ministry of Agriculture, Pirbright, Woking, Surrey.

Cairns, Dr H. J. F., Virus Research Institute, P.O. Box 49, Entebbe, Uganda, East Africa.

CARR, Dr J. G., Poultry Research Centre, West Mains Road, Edinburgh, 9.

CHANTRENNE, Dr H., University of Brussels, Laboratory of Animal Morphology, 1850 Chaussée de Wavre, Brussels, Belgium.

Chu, Dr H. P., Department of Animal Pathology, Milton Road, Cambridge.

Czekalowski, Dr J. W., Department of Bacteriology, School of Medicine, Leeds, 2.

Dickinson, Dr Loïs, Boots Pure Drug Co. Ltd, Division of Microbiology, The Priory, Thurgarten, Nottingham.

Dmochowski, Dr L., Department of Experimental Pathology & Cancer Research, School of Medicine, Leeds, 2.

Dolby, Dr D. E., Lister Institute, Elstree, Herts.

Felix, Dr A., Central Enteric Reference Laboratory & Bureau, Colindale Avenue, London, N.W. 9.

FILDES, Sir Paul, Sir William Dunn School of Pathology, South Parks Road, Oxford.

FLEWETT, Dr T. H., School of Medicine, Leeds, 2.

FULTON, Dr Forrest, London School of Hygiene and Tropical Medicine, Keppel Street, London, W.C. 1.

GARD, Professor S., Department of Virus Research, Caroline Institute, Box 764, Stockholm 1, Sweden.

Henderson, Dr W. M., Research Station, Ministry of Agriculture, Pirbright, Woking, Surrey.

Hewitt, Dr L. F., Serum Institute, Woodmansterne Road, Carshalton, Surrey.

Hotchin, Dr J. E., National Institute for Medical Research, The Ridgeway, Mill Hill, London, N.W. 7.

HOYLE, Dr L., Pathological Laboratory, General Hospital, Northampton.

Isaacs, Dr A., National Institute for Medical Research, The Ridgeway, Mill Hill, London, N.W. 7.

JOKLIK, Dr W. K., Sir William Dunn School of Pathology, South Parks Road, Oxford.

KAY, Dr D., Sir William Dunn School of Pathology, South Parks Road, Oxford.

Kleczkowski, Dr A., Rothamsted Experimental Station, Harpenden, Herts.

LATARGET, Dr R., Pasteur Laboratory of the Radium Institute, 26 rue d'Ulm, Paris, V.

LURIA, Professor S. E., Department of Bacteriology, University of Illinois, Urbana, Illinois, U.S.A.

LWOFF, Dr A., Pasteur Institute, 25 rue du Dr Roux, Paris, XV.

Macfarlane, Dr Marjorie, Lister Institute, Chelsea Gardens, London, S.W. 1.

MARKHAM, Dr R., Molteno Institute, Cambridge.

Mittler, Dr T. E., Agricultural Research Council Unit for Insect Physiology, 34a Storey's Way, Cambridge.

PIRIE, Mr N. W., Rothamsted Experimental Station, Harpenden, Herts.

Salaman, Dr M. H., Cancer Research Department, London Hospital, E. 1.

SANDERS, Dr F. K., Department of Zoology & Comparative Anatomy, Oxford.

Smith, Dr H. Williams, Animal Health Trust, Houghton Grange, Houghton, Huntingdon.

Stent, Dr G. S., Pasteur Institute, 25 rue du Dr Roux, Paris, XV.

Thung, Professor T. H., Institute for Plant Pathological Research, Binnenhaven 4a, Wageningen, Holland.

Watson, Dr J. D., Cavendish Laboratory, Free School Lane, Cambridge.

Welsch, Professor M., University of Liège, 7 rue des Bonnes Villes, Liège, Belgium.

Zinnemann, Dr. K., Bacteriological Department, School of Medicine, Leeds, 2.

CONTENTS

'ibutors v

rs' Preface ix

)rganization of the Symposium xi

HANTRENNE:
›blems of Protein Synthesis 1
)iscussion by:—HEWITT, PIRIE and BAUER.

BAWDEN and N. W. PIRIE:
us Multiplication Considered as a Form of Protein
›ynthesis 21
'iscussion by:—ANDREWES, BACKUS, STENT, SALAMAN, WATSON and THUNG

BAUER:
tabolic Aspects of Virus Multiplication . . . 46
'iscussion by:—PIRIE, CAIRNS (and ISAACS), MACFARLANE, HOYLE and KLECZKOWSKI.

MARKHAM:
:leic Acids in Virus Multiplication 85
'iscussion by:—BAWDEN, PIRIE, BOYD and BACKUS.

LURIA:
Analysis of Bacteriophage Multiplication . . . 99
'iscussion by:—WATSON, KAY, HEWITT, PIRIE, DOLBY and HOTCHIN.

<. BOYD:
: Role of Mutation in the Survival and Multiplication of
'acterial Viruses 119
'iscussion by:—LWOFF, ANDERSON, DICKINSON and BAWDEN.

ŁÉ LWOFF:
: Nature of Phage Reproduction 149
iscussion by:—WELSCH, WATSON, ALBERT, HEWITT, WILLIAMS SMITH and STENT

.TARJET:
: Multiplication of Bacterial Viruses Studied by Radio-
iological Methods 175

P. FILDES, D. KAY and W. K. JOKLIK:
Divalent Metals in Phage Production 194
Discussion by:—PIRIE.
FELIX:—Bacteriophage not a virus?

SVEN GARD:
Aspects of the Formation of Incomplete Virus . . . 211
Discussion by:—HOYLE, ANDREWES, BAUER and BAWDEN.

L. HOYLE:
The Multiplication of the Influenza Virus considered in Relation to the General Problem of Biological Multiplication 225
Discussion by:—GARD, CAIRNS and BROOKSBY.

T. H. FLEWETT:
Growth of Viruses of the Influenza Group as Revealed by Electron Microscopy of Infected Cells 249
Discussion by:—PIRIE and SALAMAN.

FORREST FULTON:
The Argument for a Venerable Hypothesis of Virus Multiplication 261

G. H. BERGOLD:
The Multiplication of Insect Viruses 276
Discussion by:—FELIX, MITTLER, PIRIE, CHU, ZINNEMANN and DMOCHOWSKI.

J. G. CARR:
The Multiplication of the Fowl Tumour Viruses . . 284
Discussion by:—DMOCHOWSKI and LATARJET.

F. K. SANDERS:
Possible Multiplication Cycles in Neurotropic Viruses . 297
Discussion by:—BAWDEN, HENDERSON and CZEKALOWSKI.

EDITORS' PREFACE

Members of the Symposium will no doubt notice that the account of the discussions of papers in this volume is uneven.

Many questions and criticisms went unrecorded on the forms supplied, or did not reach the Editors. Further, some of those which came in had been polished and made more cogent. The effect of this was that the replies already on hand were either irrelevant or ineffective.

Under these circumstances it was clear that the Editors were unable to provide a factual report of the proceedings, and they decided to base an account on ideal conditions in which the questions and answers could be well considered. In this way, they thought, the scientific, rather than the personal aspects of the discussion, might be registered more satisfactorily. As the ideal questions or criticisms were received, they were forwarded to the author of the paper discussed. He thus had the advantage of the last word. This procedure also allowed acceptance of questions which, so far as the Editors could remember, had never been asked at all.

It was found possible to collect and circulate all these documents and to recover corrected proofs of the main papers and all replies to discussions within four weeks of the termination of the Symposium.

In a subject in which movement in discovery is so rapid, four weeks is, of course, a long time and this led to such alterations in proof as normally could not be allowed. It also permitted at least one author to carry out further experimental work to clarify a matter which the Symposium had left obscure. The Editors decided that even in this case a postdated 'stop press' note came within the intentions of the Symposium.

This volume thus contains the final versions of the main contributions and, attached to each, the 'discussion' in an idealized form.

Sir William Dunn School of Pathology PAUL FILDES
University of Oxford W. E. VAN HEYNINGEN
17 *June* 1952

THE ORGANIZATION OF THE SYMPOSIUM

The Committee of the Society for General Microbiology having selected for a Symposium a controversial subject, it became necessary to organize it in such a way as to ensure that an even balance was maintained between opposing factions, and that their respective views should be exchanged and conveyed to the prospective audience in advance of the meeting so that the discussion should be conducted as effectively as possible. About eight months before the event the Society invited a representative group of virus experts to write a full account of their views. These were set up in type, and about one thousand sets of galley proofs were distributed to all members of the Society three weeks before the opening of the Symposium. The meeting, which took place at Oxford University on the 16th and 17th of April, 1952, was attended by some 400 persons. The galley proofs were taken as read and, after brief introductions by the authors, the papers were thrown open to discussion by invited speakers and the audience generally. Each speaker was provided with a printed form on which his remarks, whether questions or answers, were to be recorded and handed in for publication.

The organization of the Symposium on these lines entailed considerable expenditure. A large proportion of the cost of printing the galley proofs was met by a contribution of £250 from the Nuffield Foundation. Further printing costs and travelling expenses of visitors invited from abroad were met by subscriptions amounting to £710 10s. 0d. from the following organizations: Allen & Hanbury's Ltd, Boots Pure Drug Co. Ltd, The Distillers Co. (Biochemicals) Ltd, Evans Medical Supplies Ltd, Glaxo Laboratories Ltd, May & Baker Ltd, Pest Control Ltd, The Wellcome Foundation. The Society is glad to record here its gratitude for this generous support, without which it would not have been possible to run a successful Symposium.

W. E. van Heyningen

PROBLEMS OF PROTEIN SYNTHESIS

H. CHANTRENNE

University of Brussels, Laboratory of Animal Morphology

A FEW FUNDAMENTAL DATA

Two proteins having the same physiological function in two different species of animals are often different enough to be easily distinguished by chemical analysis, solubility measurements or other relatively crude physical or chemical tests. Closely related strains of the same micro-organisms can be identified by small differences in their metabolism, i.e. in their enzymatic equipment, and the study of biochemical mutants in micro-organisms (Beadle, 1945; Bonner, 1946) or in higher organisms (Perutz *et al.*, 1951) has shown, in many instances, that mutations correspond to the loss or to the modification of one type of protein. The formation of a given protein in a cell thus depends on the genetic characteristics of that cell.

On the other hand, the discovery of the acquisition of new enzymes by micro-organisms grown in a new medium and the very careful analysis of this phenomenon, has shown that the actual formation of an adaptive enzyme depends on both genetic characteristics and external conditions (Spiegelman, 1946, 1949; Monod, 1948, 1950; Ephrussi & Hottinguer, 1950). A given adaptive enzyme can form only in cells possessing a suitable genotype, and its actual formation requires the presence of the corresponding substrate. Another example of the action of external factors on the production of specific proteins is the formation of antibodies specific for the antigens which caused their production.

The proteins in a non-growing cell have been considered for a long time to be stable and inert; but the pioneer work of Schoenheimer (1942) on the incorporation of labelled amino acids into the proteins has completely changed that picture, by showing clearly that the substance of the proteins in the living cell is in a dynamic state, being continuously replaced by that of small molecules of the environment. Innumerable works have confirmed this for many various types of cells. This is thus a fundamental fact that must be kept in mind in any consideration of the mechanism of protein synthesis.

The study of viruses has established two basic facts, among many others. First, that the introduction of the virus into a receptive cell causes this to produce a group of proteins that the non-infected cell would never have produced, as if the presence of a virus particle had caused the cell mechanisms to reduplicate that particle manyfold. Second, virus studies have shown that these 'self-duplicating' particles contain a large amount of nucleic acid, be it ribosenucleic or desoxyribosenucleic. Other known self-duplicating particles have also been shown to contain nucleic acids. Thus chromosomes contain both types, chloroplasts contain RNA, and there are reasons to think that the self-duplicating particles responsible for enzyme production also contain ribosenucleic acid (Spiegelman, 1949).

RNA is most probably involved not only in the self-duplicating processes, but also in protein synthesis in general. The histological and chemical studies on the distribution of RNA in different tissues by Brachet (1941, 1947) and the microspectrophotometric researches of Caspersson *et al.* (1941) have led to the recognition of a constant coincidence in space and time of a high content of RNA and rapid protein formation. At the time when, and in the place where, proteins are being rapidly formed, the amount of RNA present is relatively high. This relation is particularly striking when the same tissue is considered under two different physiological conditions (Jeener, 1951). For instance when protein formation or growth is taking place the RNA content is high, but when the same cells are not producing proteins, there is less RNA. The number of facts all pointing in the same direction is very impressive, and the great number of experiments reported since that relation was first discovered have largely confirmed the existence of a correlation between RNA and protein synthesis (Brachet, 1950). Moreover, there is evidence that ribosenucleic acid accumulation occurs before protein synthesis (Thorell, 1947; Brachet, 1950; Davidson *et al.*, 1949).

The transformation of one type of bacterium into another by purified DNA (Avery *et al.*, 1944; Boivin *et al.*, 1945; Hotchkiss, 1948) also suggests an effect of DNA on the structure of proteins synthesized, since we are accustomed to consider a change in metabolism or constitution as the reflection of a difference in enzyme equipment. The formation of galactozymase in adapted yeasts can be inhibited by ultraviolet irradiation, and it is very impressive that the action spectrum for this phenomenon closely resembles a nucleic acid absorption spectrum (Swensson, 1950). It has also been shown, that the turnover of RNA phosphorus in yeast is several times higher when the cell is producing proteins, and that several factors inhibiting protein formation also inhibit the replacement of the RNA phosphorus (Spiegelman & Kamen, 1947). Quite recently, Hultin (1950) working on the liver of chick embryo, Keller (1951) and Lee *et al.* (1951) on rat liver, and Szafarz (1951) on a flagellate, have observed that the rate of incorporation of amino acids into proteins is very different for various particulate fractions that can be isolated by centrifugation of the homogenized tissues. The highest speed of amino acid ncorporation was found in the proteins of the 'microsomes' and especially in the smallest of those particles which are known to have the highest RNA content of all the cell particles. The larger particles containing he respiratory enzymes and the centres of high energy phosphate bond production were much less active in this respect. This is true also for the ncorporation in homogenates (Borsook, 1950; Siekevitz & Zamecnik, 1951). These results extend to the cell particles the previously recognized coincidence of high nucleic acid content and protein synthesis, and may be taken as good evidence that the microsomes are a site of protein formation (Brachet, 1947).

Recent comparative studies on the activity of the cytoplasm in the presence or absence of the nucleus, have confirmed the conclusion that protein synthesis is essentially a cytoplasmic function. *Acetabularia mediterranea* is a large mono-cellular alga that can be cut into two halves one of which contains the nucleus, while the other is free. Both halves will survive for several weeks. It has been observed (Brachet & Chantrenne, 1951) that the rate of incorporation of $^{14}CO_2$ into the proteins of nucleated and unnucleated halves of the algae remains the same for two weeks after division. After thirty-eight days, the rate of incorporation into the proteins of the unnucleated halves still amounted to 70% of that in the nucleated halves. This indicates that the complete system for amino acid incorporation into proteins is cytoplasmic, and that the action of the nucleus on this process is indirect and remote.

The same conclusion has been reached in an interesting study (Jeener & Jeener, 1952) on the behaviour of a thermobacterium grown in the absence of either uracil, which limits RNA synthesis, or of desoxyribonucleosides, which limit DNA formation. Growth appears to be related directly to the formation of RNA, but much more remotedly to the formation of the DNA of the nucleoids.

The fascinating work on enzymatic adaptation, and on mutations caused by acriflavine in yeast (Ephrussi) indicates that adaptive enzymes and even such a fundamental enzyme as cytochrome oxidase, are indeed formed in the cytoplasm, where they are produced by self-duplicating particles. In turn the duplication of these particles is controlled by genetic factors segregating in accordance with Mendelian laws. (Chen *et al.*, 1950; Spiegelman, 1946; Monod, 1948).

Direct biochemical studies on protein formation have been difficult so far. Net protein synthesis *in vitro* has been obtained in a few cases.

Peters & Anfinsen (1950) have obtained serum albumin formation by slices of chicken liver. They found that the respiration of the slice, and the integrity of its mechanisms for producing high energy phosphate substances were necessary for albumin synthesis. Hokin (1951*a*) observed the *in vitro* formation of amylase by pancreas slices; in this tissue also respiration producing high energy phosphate bonds was required. Again Hokin (1951*b*) made the very important observation that the addition of the essential amino acids to the incubation medium enhances the amylase production. Methionine however has no effect. This is an interesting fact indeed, since amylase does not contain any methionine. Thus, the net formation of a protein responds very rapidly to the presence of its constituent amino acids.

Tracer work on the incorporation of the amino acids into the proteins of slices or of homogenates of various animal tissues (review by Borsook, 1950) also emphasizes the necessity of high energy phosphate bonds for the incorporation of the amino acids, with the possible exception of lysine. When several amino acids are incorporated simultaneously by a given system, there seems to be no mutual interference, and the amino acids are incorporated independently (Borsook *et al.*, 1950). This last result is apparently at variance with the requirement for all the amino acids in a net synthesis as observed by Hokin (1951*b*) and especially apparent from feeding experiments in which it has been conclusively shown that the essential amino acids are not able to sustain growth or even nitrogen balance in an animal when they are fed at intervals of, e.g. two hours, whereas they are effective when fed simultaneously (Geiger, 1950).

This brief review of some major experimental facts shows that in spite of a fairly large accumulation of data, we are still far from understanding the mechanism of protein production. In the rest of this paper, we are going to examine a few of the main questions that are still to be solved, and discuss the present state and perspectives of different aspects of the problem of the mechanism of protein biosynthesis.

PEPTIDE BOND FORMATION

There is very little information about possible intermediate polypeptides or lower peptides between the free amino acids and the proteins, and so far it has not been possible to study separately the formation of a part of a protein. Most of the information now available on the condensation of amino acids is derived by analogy with somewhat comparable and simpler systems in which peptidic linkages are formed.

For instance, the formation of the CONH bond in acetylamines, hippuric acid, glutamine and glutathione have been extensively studied. There is one common feature of all these reactions, the synthesis depends on high energy phosphate compounds. The case of acetylamines has been elucidated by the investigations of Lipmann and his coworkers. In pigeon liver, acetylsulfonamide is formed in the following way: adenosinetriphosphate (ATP) and acetate react enzymatically with coenzyme A, with the formation of acetyl coenzyme A, phosphate and ADP. The acetyl coenzyme A then reacts with the amine in the presence of another enzyme; this results in the formation of the acetylamine, with regeneration of coenzyme A. The mechanism of this peptide bond formation thus essentially consists in the activation of the carboxyl group at the expense of high energy phosphate, followed by the transfer of the acetyl radical via coenzyme A to the amino group, which does not undergo, as it seems, any preliminary transformation, except its fixation on the enzyme.

Hippuric acid formation from benzoate and glycine is probably dependent on a very similar process, since coenzyme A is again involved (Braunstein, 1950; Chantrenne, 1950). On the other hand, coenzyme A is not required for glutamine synthesis (Speck, 1949) and it is inhibitory for the incorporation of labelled glycine into glutathione (Bloch, 1951). In glutamine synthesis, it is clear that the γ-carboxyl group of glutamic acid is activated at the expense of ATP, and it has been suggested that the intermediate might be γ-glutamylphosphate, but this is not established. Recently Bloch (1951) found an unknown coenzyme to be involved in amino acids incorporation into glutathione, and developments in this field will be watched with great interest, for the cysteine—glycine bond in glutathione is the only true α-peptide linkage between two amino acids, the formation of which has been studied so far. It might provide the best model of peptide bond formation.

The cases of peptide bond formation we have just described can be considered, at best, as models of a mechanism of peptide synthesis, but there is no evidence that similar processes play a part in protein formation. However, the experimental fact that protein synthesis depends on phosphorylation suggests at least a possible analogy with the simpler cases of peptide linkage formation, and allows one to take as a working hypothesis that high energy phosphate compounds serve to activate the carboxyl groups of amino acids prior to condensation (Lipmann, 1941).

This is not the only possibility though, and it is quite conceivable that the individual amino acids are not activated directly at the expense of high energy phosphate. It is possible that a few types of peptide bonds only, e.g. those of glutathione or glutamine, are made at the expense of phosphorylation mechanisms and then 'transferred' to other peptides by transpeptidation (Linderstrøm-Lang, 1949; Fruton, 1950, 1951; Virtanen, 1950).

Enzymes catalysing transpeptidation reactions have been found in various tissues, but little information has been obtained so far concerning their significance for polypeptide and protein formation. It is our opinion that two types of transpeptidation reactions may be distinguished among the data of the literature: transpeptidations catalyzed by proteolytic enzymes and transpeptidations catalyzed by enzymes that might be called 'non-hydrolyzing transpeptidases'. It is clearly established that pure proteolytic enzymes like chymotrypsin or papain catalyze the direct replacement of one of the members of a peptide by another molecule (Behrens & Bergmann, 1939; Johnston *et al.*, 1950*b*; Fruton, 1951) but at the same time the same enzymes catalyze the hydrolysis of all the peptides present, including the newly-formed peptides. There is a competition between water and the amine for the carboxyl group at the level of the enzyme, and this competition is bound to be decided in favour of water, since the hydrolysis equilibrium for all the peptides is far to the side of complete hydrolysis. It seems to us that such transpeptidations have little chance of playing an important part in polypeptide synthesis in the living cell unless, for some reason, water could not enter the competition, i.e. unless the functional enzymes *in vivo* were not hydrolyzing. The same remarks apply to the transfer of the carboxyl group of an ester onto the amino group of an amino acid, that has been shown to be catalyzed by proteolytic enzymes (Brenner *et al.*, 1950, 1951).

The other type of transpeptidation is exemplified by the glutamyl-transferase of Stumpf & Loomis (1950) and possibly also of Hanes *et al.* (1950) and Grossowicz *et al.* (1950). An enzyme isolated from fruit seeds catalyzes the replacement of the γ-NH_2 group of glutamine by -NHOH when the enzyme is incubated with glutamine, hydroxylamine, Mn ions and a catalytic amount of ATP. The process is very different from the other type of transpeptidations. In the present case there is *no hydrolytic cleavage* of glutamine or of the hydroxamic acid formed and the initial γ-glutamyl group is transferred, without loss, in a stoichiometric reaction (Stumpf, 1950). This type of reaction might of course operate with a high yield and should be considered as a model of reactions that might be involved in polypeptide formation. The transglutamylations observed by Hanes and co-workers (1950) between glutathione and various amino acids might be of a similar type. It seems probable that the enzyme splitting the γ-glutamyl bond of glutathione is not a hydrolytic enzyme but a transglutamylase that will transfer the glutamyl group from the NH_2 group of cysteinylglycine to that of various amino acids. A point that may be very important, and that as far as we know has not been stressed, is that a second enzyme associated with the first (Olson & Binkley, 1950), and present in Hanes's system, hydrolyzes cysteinylglycine very rapidly, although it will not touch glutathione. The result of this is that glutathione completely disappears, one of its peptide bonds being transferred to a new peptide (e.g. γ-glutamylvaline) and the other hydrolyzed. If we consider the equilibrium of the two reactions we can see that the splitting of cysteinylglycine shifts the equilibrium of the first reaction towards the right, and that the two reactions are coupled.

$$\gamma\text{-glutamylcysteinylglycine} + \text{valine} \rightleftharpoons \gamma\text{-glutamylvaline} + \text{cysteinylglycine}$$
$$\text{cysteinylglycine} + H_2O \rightarrow \text{cysteine} + \text{glycine}$$

Glutathione in such a system might function as a very efficient γ-glutamylating agent. The net yield in new peptide bond formed is only 50% if the first enzyme is not hydrolytic, but the new glutamylpeptide is stable, for the equilibrium of the total reaction is just as much in favour of the formation of the new glutamylpeptide as it is in favour of the free amino acids when a peptide is exposed to proteolytic enzymes. This seems to be an example of coupling of hydrolysis to synthesis of peptide bond that is reminiscent of a mechanism suggested by Bergmann & Fruton (1944).

Before closing this section, let us remember that carboxyl activation of amino acids and transpeptidations are not more, at present, than suggested models of peptide formation and that the actual mechanism involved in protein formation is not known.

ARRANGEMENT OF AMINO ACIDS INTO POLYPEPTIDE CHAINS

The formation of a given polypeptide chain raises a second question, namely the mechanism of the selection of the amino acids from which the peptide bonds will be formed. At present we have no reason to believe that there exists any regularity of arrangement of the individual amino acids in the chains. With the refinements of analytical methods the idea of a periodicity of the amino acids had to be given up, and when examining the arrangement of the amino acids in a chain of insulin, as revealed by the outstanding work of Sanger (1951), one cannot indeed find any regularity or periodicity; the amino acids are arranged apparently in the most arbitrary order. However we are bound to consider that they are arranged in a definite order and not distributed at random in the chains, otherwise a research like Sanger's would have led to no consistent result, and the constant biological and physico-chemical properties of insulin would be impossible to account for.

If the amino acids are not distributed at random in the protein molecule, then there must be some mechanism in the cell which causes a certain arrangement to be repeatedly and consistently produced. For each protein a unique arrangement is formed among the very great number of isomers or homologues one can think of. We know nothing about the actual mechanism involved, but several suggestions have been proposed; let us examine some of them and see to what extent they are supported by facts.

It is possible that amino acids are first activated at the expense of ATP and then united in polypeptides under the action of a series of enzymes that have not yet been discovered. This is evidently hypothetical and is based only on the necessity of phosphorylation for protein synthesis.

Following the discovery of the specificity of proteolytic enzymes for some arrangements of amino acids, and their ability to catalyse transpeptidation, it was suggested that a given assortment of proteolytic enzymes might organize the peptide chains by a series of transpeptidations (Behrens & Bergmann, 1939). Simple rearrangements of this type have actually been observed (Fruton, 1950; Waley & Watson, 1951). However they suffer from the unfavourable competition with water. This makes it rather doubtful that such systems might operate efficiently for protein synthesis, unless the properties of the enzymes involved *in vivo* be somewhat different.

We would like to suggest a related but different possibility for the selection of arrangements by the proteolytic enzymes. If peptides were built by transpeptidation of the Stumpf type, and if after each transpeptidation specific proteolytic enzymes would *hydrolyse* a bond of one of the peptides just formed, the specific attack on, and the splitting of the 'forbidden arrangement', would stabilize the other arrangement by making the transpeptidation complete. This system of selection is merely a generalization of the system observed in the transpeptidations with glutathione, in which the 'forbidden arrangement' is cysteinylglycine.

In any of the mechanisms just considered, many enzymes would be involved in the building up of a polypeptide chain. It should be realized, however, that the production of a protein with a definite arrangement of amino acids raises a question that does not appear in the formation of the other constituents of living systems. The specificity of any biochemical process is always explained, correctly we think, by the specificity of the enzyme or of the many enzymes involved in a chain of successive reactions. But when the formation of a protein is considered the question as to the number of enzymes involved in the determination of the specificity of that protein becomes acute; for the problem cannot be solved this time by simply referring to the specificity of the enzymes involved. This would amount to displacing the problem, for these enzymes are also specific proteins and they are also made by the same cell. It is not known at present whether many proteins, in a given cell, are formed from a relatively small number of larger building blocks combined in various ways, or whether the arrangements of the amino acids in the different proteins produced have no relation to one another. But even if a deeper knowledge of the composition and of the comparative anatomy of the protein molecules should reveal that certain sequences of amino acids are common to many proteins, it would remain that for each protein there must exist at least one agent orienting the synthesis towards the production of the particular protein considered, beside the several enzymes doing the common work of polypeptide building. We would again be faced with the difficult situation that we explain the formation of a protein by that of more proteins. This difficulty would be less if the orientation of protein synthesis was determined by a protein identical with the protein synthesized, or by a protein or non-protein substance having a complementary structure to that of the protein synthesized.

Thus we are led to consider another possibility of determination of the specificity, namely the arrangement of the building blocks into the right order under the action of models or templates. The idea of a template mechanism has been the subject of many speculations and it fits rather well with experimental facts that are difficult to explain otherwise, e.g. the fundamental observation of virus reproduction, that the introduction into a cell of the foreign nucleoproteins of the virus completely changes the orientation of the synthesizing mechanisms of the cell, and causes it to produce nucleoproteins identical to those of the sample introduced. Thus, the virus has brought into the cell all that is necessary for orienting the synthesis towards the building of precisely its own components, i.e. it has brought into the cell models to be copied.

The formation of antibodies specific for the antigen injected is another striking experimental fact that can be interpreted easily in terms of a template process (Breinl & Haurowitz, 1930; Pauling, 1940). The same is true for adaptive enzymes formation, and for the genetic control of protein formation.

If templates are a reality they must be found in the chromosomes, in the genetic units of the phages, in the viruses, in all the particles endowed with genetic continuity and probably in microsomes. Our present knowledge of some of these particles makes it probable that the templates, if there are such things, are proteins, nucleoproteins, or nucleic acids. Some good discussions on possible template mechanisms may be found in the recent literature (Haurowitz, 1949, 1950) and it is not our intention to discuss them here. However we would like to mention only one debated point, namely the idea that the replica of a template would be necessarily the image of the template, so that the process should involve the formation of two series of structures, one being the negative print of the other. This does not necessarily follow; for instance the structure of unfolded proteins as suggested recently by Pauling & Corey (1951) and named the 'pleated sheet', would give a contact copy identical to itself.

THE FOLDING OF THE POLYPEPTIDE CHAINS

The physical properties of the proteins, the X-ray diffraction data, and the changes of properties accompanying the hydrolysis or the denaturation of proteins have indicated that polypeptide chains are folded in a certain structure held together by hydrogen bonds and possibly some salt-like linkages. The particular folding of the chains is very important for the properties of the proteins, since enzymes lose their catalytic properties when their structure is modified (denatured). Most interesting is the case of 'reversible denaturation' in which it has been shown that e.g. trypsin can be inactivated by heat but that it will spontaneously return to its 'native' state if the temperature is lowered. In this case at least, the peptide chain can probably recover its normal folding after this has been disturbed to a certain extent by external agents. However, this is not the case for all the proteins, and many will not return to their native state. After denaturation by heat the chains will be bound to one another in different ways, and the whole substance may flocculate.

In a hypothetical solution of unfolded free polypeptide chains there is very little chance that all the individual chains would spontaneously fold in the same way into discrete and identical structures. But it is quite an assumption to imagine that polypeptide chains are first made, and then folded. If they 'grow' by binding amino acids at one or both ends the folding may start from the very beginning of the process, and each step of the folding may be a necessary consequence of the preceding step of synthesis. If polypeptide chains are formed on a template this has certainly an effect on the folding of the chain.

We know entirely too little about this process at present to know even whether the folding raises a problem similar to that of the arrangement of the amino acids, or whether it is a mere consequence of this arrangement.

THE ROLE OF NUCLEIC ACIDS

Although the idea that nucleic acids play a part in protein synthesis is supported by many observations and data we are still very ignorant about the mechanism of its action.

There is little doubt that nucleic acids are necessary components of self-duplicating particles showing genetic continuity, i.e. the reproduction of a certain organization at the molecular and particle level. Some years ago, nucleic acids were considered as rather simple structures offering very few possibilities of diversity. But recent developments of methods of purification and analysis have resulted in the recognition of the fact that nucleic acids are much more complex, and it seems not unreasonable to consider that there might be as great a variety of nucleic acids as of proteins. The great specificity, that has been considered a unique feature of the proteins only, is probably shared by nucleic acids. The nucleic acid nature of the transforming agents of pneumococcus and other bacteria is probably the most convincing evidence of nucleic acid specificity. As a result the status of the two main constituents of particles with genetic continuity appears now much more balanced than before. The respective parts played by nucleic acids and proteins in the reproduction of such particles is not well understood, but it seems that the study of virus reproduction has gone far towards the elucidation of this problem. This will certainly be discussed by specialists in this symposium.

Aside from the self-duplicating particles, what is the role of ribosenucleic acid in the production of proteins? There are indications that proteins, enzymes at least, are produced by self-duplicating particles (Spiegelman *et al.*, 1950; Ephrussi & Hottinguer, 1950). On the other hand, the microsomes seem to be the site of rapid incorporation of amino acids into the proteins (Borsook, 1950; Keller, 1951; Siekevitz & Zamecnik, 1951; Szafarz, 1951). Thus it may be that the relation between concentration or localization of RNA and active protein synthesis is due to the abundance of protein-synthesizing units, which contain RNA, and that the duplication of these particles preceding a burst of protein synthesis is responsible for the increase in RNA (Brachet, 1944, 1947, 1949).

Several papers on the comparison of the rate of glycine incorporation into proteins and into the purines of RNA have been published recently. Hammarsten's group (1951) found a constant relation between the incorporation of glycine into proteins and RNA in growing tissues, and very little incorporation both into RNA and into the proteins of resting yeast cells. Also, glycine is incorporated into RNA earlier than into proteins, which would indicate that RNA synthesis occurs before protein synthesis, if it is assumed that the rate of incorporation reflects the speed of synthesis. On the other hand Abrams (1951*b*) found that it is possible to inhibit the incorporation of phosphate into RNA by X-ray irradiation, without greatly affecting protein synthesis.

It is our opinion that it is very difficult to draw conclusions at present from these experimental data, for three main reasons. First it is known that different proteins have different turnover rates in the same organ (Sprinson & Rittenberg, 1949) and this is true also for ribosenucleic acid (Jeener, 1949, 1950; Barnum, 1950). Second, in view of the problems considered here, it would be very important to distinguish between the proteins that are components of the self-duplicating particles and the other proteins of the cell. Third, the meaning of the rate of incorporation of a labelled building block into a larger molecule is not clear. For instance the fact that in non-growing tissues phosphate incorporation into DNA is very slow (Brues, 1949; Hammarsten, 1951; Jeener, 1949) whereas the incorporation of glycine into DNA is much faster (Le Page & Heidelberger, 1951), makes it clear that it is very dangerous to consider the rate of incorporation of a small group into a large molecule as a measure of the speed of synthesis of this large molecule (see also Abrams, 1951*a*). These discrepancies might be due to our ignorance of the real precursor in the different cases, but it may also be due to the existence of reversible enzyme catalyzed reactions bringing about exchanges of groups even when net synthesis does not occur.

If we wish to consider the role of RNA or DNA at the level of chemical reactions we are left with nothing but hypothesis. Nucleic acids, being an essential part of self-duplicating particles, have been considered as a fundamental piece of the copying mechanism. It has been suggested, for instance, that nucleic acid is a replica of the protein molecule in the sense of a complementary distribution of electric charges (Friedrich-Freksa, 1940; Rondoni, 1940), or that it is the backbone holding the template protein in the unfolded form (Haurowitz, 1949, 1950). Spiegelman (1949) has proposed also that it is an organized structure able to deliver the energy for peptide bond synthesis at the proper place, e.g. a bearer of anchored phosphoric acid groups on which the carboxyl groups of amino acids are being activated by acylphosphate formation (Chantrenne, 1950). This is all very speculative and the experimental facts are too scarce at present to allow either a choice to be made or any possibility to be discarded.

THE MEANING OF THE DYNAMIC STATE OF THE PROTEINS

It is generally assumed that the incorporation of labelled amino acids into the proteins of non-growing tissues is evidence that in the cells two separate and competitive processes are working simultaneously. One process is the condensation of amino acids into proteins, and the other their degradation by proteolytic systems. The question as to whether net synthesis, maintenance or net degradation takes place is supposed to be a matter of who wins the race (Rittenberg & Shemin, 1946). This might indeed be true. It should be realized, however, that this scheme is not the only possible interpretation of the experimental fact. The experiments just show that individual molecules of amino acids that were free in the medium at time *o* are found as constituents of proteins at time *t*. But this does not decide whether the amino acid molecules considered (labelled) are contained in thoroughly new protein molecules as is generally assumed, or whether they have just replaced identical amino acids that were part of the protein molecule present at time *o*.

Generally speaking, incorporation of a labelled group into a substance can be the result of a net synthesis, or of the enzyme catalyzed exchange of the non-labelled group for the corresponding labelled group. For instance, when pyruvate is incubated with certain Coli extracts in the presence of formate and coenzyme A, very little if any change in concentrations occurs. However, if ^{14}C labelled formate is used, it can be observed that the carboxyl group of pyruvate very rapidly incorporates the formate carbon and that an equal distribution of isotopes is reached within a short time (Chantrenne & Lipmann, 1950). Adopting the same vocabulary as in the case of protein, we would say that pyruvate is in a dynamic state in the presence of the Coli enzyme, which is a correct statement of the fact. But it would be misleading to state that pyruvate is continuously being built up and broken down by two competing mechanisms. In fact, there is only one reversible reaction, and the pyruvate molecules in the environment of the enzyme from time to time are bound by the enzyme and activated. That is, the pyruvate is fixed on the enzyme in such a way that the—CO—COOH linkage is labilized. The CH_3CO-radical when bound to the enzyme can combine with any formyl group that happens to be fixed on the enzyme at the same time. This process is going on all the time and it would occur whatever the direction of the net reaction, even if the total concentration of pyruvate was decreasing.

Such exchange reactions are well known, but in general they have not been considered seriously for explaining amino acid incorporation into proteins (see however Borsook, 1950). This may be the result of the difficulty in visualizing how amino acids, buried within a long polypeptide chain, would exchange with free amino acids. But this is not a sufficient reason to disregard the possibility of exchange. Some experimental facts are indeed difficult to account for if incorporation is considered as reflecting the continuous *one way* flow of substance through the proteins. The fact that in the absence of metabolizable substrate or in the presence of inhibitors of its synthesis, galactozymase does not disappear from adapted yeasts, is difficult to explain in terms of two different and competitive processes. It is indeed surprising that the hydrolysis is precisely inhibited by the same agents which inhibit synthesis (Spiegelman & Nozawa, 1945). It has been shown recently (Simpson, 1951) that the *release* of labelled amino acids from liver protein is inhibited by dinitrophenol which also inhibits the incorporation. This is easy to understand if the same reaction is responsible for both incorporation and release, but rather surprising if two competitive reactions are involved. Again the independent incorporation of amino acids (Borsook, 1950) is difficult to reconcile with the requirement for all the essential amino acids at the same time (Geiger, 1950) for net synthesis, if incorporation means net synthesis.

We are of opinion that the possibility of exchanges should not be overlooked. Just as pyruvic acid or saccharides (Doudoroff, 1947) are known to be labilized by enzymes in such a way that their constituent groups can be rapidly exchanged, we might have to face the possibility that proteins might be able to go on and off some active surface ('template') on which they were built, and that when they are on the 'template' their constituent amino acids might be exchanged independently. This amounts to extending to the case of 'templates' the properties that are familiar for ordinary enzymes, or to considering the template as a polyvalent enzyme on which all the peptide bonds of a polypeptide chain are labilized.

Essentially this is nothing more than an alternative interpretation of the dynamic state of the proteins, but it is quite different from the generally accepted view and it might prove to be of some heuristic value.

It is to be hoped that well-designed experiments will tell us what the actual mechanism of the dynamic state of the proteins is, for the approach to growth control is directly connected to that knowledge. If the protein turnover reflects a one-way chain of two series of reactions, one for building, the other for splitting, the problem is to understand the fine adjustment of the two mechanisms and to learn how to dissociate and control them. If on the other hand the mechanism is of the type we have outlined above, the problem is one of control of template activity and template duplication.

The centre of the problem of protein synthesis is, in our opinion, the arrangement of the amino acids, and this problem is of such a nature that we will have probably to reach new concepts of biochemical mechanisms in order to account for the observed facts. It seems to us that time is ripe for focussing the attention onto the possibility of template mechanisms, and for trying to discover how they work. Virus study has done much in this respect, and biochemists interested in the problem of protein biogenesis are eager to know more about virus reproduction, for they feel that they have probably more to learn from virus research than the virologists can expect to learn from biochemical research in this field, for the time being.

REFERENCES

ABRAMS, R.'(1951*a*). Some factors influencing nucleic acid purine renewal in the rat. *Arch. Biochem.*, **33**, 436.

ABRAMS, R. (1951*b*). Effects of X-rays on nucleic acid and protein synthesis. *Arch. Biochem.*, **30**, 90.

AVERY, O. T., MACLEOD, C. M. & MCCARTY, M. (1944). Induction of transformation by a desoxyribonucleic acid fraction from Pneumococcus Type III. *J. Exp. Med.*, **79**, 137.

BARNUM, C. P. &. HUSEBY, R. A. (1950). The intracellular heterogeneity of pentose nucleic acids, as evidenced by the incorporation of radiophosphorus. *Arch. Biochem.*, **29**, 7.

BEADLE, G. W. (1945). Biochemical genetics. *Chem. Rev.*, **37**, 15.

BEHRENS, O. & BERGMANN, M. (1939). Cosubstrates in proteolysis. *J. Biol. Chem.*, **129**, 587.

BERGMANN, M. & FRAENKEL-CONRAT, H. (1937). The role of specificity in the enzymatic synthesis of proteins. *J. Biol. Chem.*, **119**, 707.

BERGMANN, M. & FRUTON, J. S. (1944). The significance of coupled reactions for the enzymatic hydrolysis and synthesis of proteins. *Ann. N.Y. Acad. Sci.*, **45**, 409.

VON BERTALANFFY, L. (1941). Bemerkungen zum Modell der biologischen Elementareinheiten. *Naturwiss.*, **32**, 26.

BINKLEY, F. (1951). Metabolism of glutathione. *Nature*, **167**, 888.

BINKLEY, F. & OLSON, C. F. (1951). Activators and inhibitors of the hydrolysis of glutathione. *J. Biol. Chem.*, **188**, 451.

BLOCH, K. (1951). A heat stable cofactor for glutathione synthesis. *Federation Proc.*, **10**, 163.

BOIVIN, A., DELAUNAY, A., VENDRELY, R. & LEHOULT, Y. (1945). L'acide thymonucléique polymérisé, principe paraissant susceptible de déterminer la spécificité sérologique et l'équipement enzymatique des bactéries. *Experientia*, **1**, 334.

BONNER, D. (1946). Biochemical mutations in Neurospora. *Cold Spring Harbor Symp.*, **11**, 4.

BORSOOK, H. (1950.) Protein turnover and incorporation of labelled amino acids into tissue proteins *in vivo* and *in vitro*. *Physiol. Rev.*, **30**, 206.

BORSOOK, H., DEASY, C. L., HAAGEN-SMIT, A. J., KEIGHLEY, G. & LOWY, P. H. (1950). Incorporation of C^{14}-labelled amino acids into proteins of fractions of guinea-pig liver homogenates. *Federation Proc.*, **9**, 154.

BRACHET, J. (1941). La localisation des acides pentosenucléiques dans les tissus animaux et les oeufs d'Amphibiens en voie de développement. *Arch. Biologie*, **53**, 207.

BRACHET, J. (1944). *Embryologie chimique*. English ed. New York: 1950. Interscience Publ.

BRACHET, J. (1947). Nucleic acids in the cell and the embryo. *Symp. Soc. Exp. Biol.*, **1**, 207.

BRACHET, J. (1949). L'hypothèse des plasmagènes dans le développement et la différenciation. *Pubbl. Staz. Zool. Napoli Suppl.*, **21**, 77.

BRACHET, J. (1950). Synthèse des protéines et ribonucleoproteides. *Exposés ann. Biochem. méd.*, 12 sér. Paris: Masson.

BRACHET, J. & CHANTRENNE, H. (1951). Protein synthesis in nucleated and non-nucleated halves of *Acetabularia mediterranea* studied with carbon-14-dioxide. *Nature*, **168**, 950.

BRAUNSTEIN, A. E. & YEFIMOCHKINA, E. F. (1950). Effect of pantothenic acid deficiency on hippuric acid synthesis in the rat. *Doklady Akad. Nauk. S.S.S.R.*, **71**, 347.

BREINL, F. & HAUROWITZ, F. (1930). Chemische Untersuchung des Präzipitates aus Hämoglobin und Anti-Hämoglobin Serum und Bemerkungen über die Natur des Antikörper. *Z. physiol. Chem.*, **192**, 45.

BRENNER, M., MÜLLER, H. R. & PFISTER, R. W. (1950). Eine neue enzymatische Peptidsynthese. *Helv. Chim. Acta*, **33**, 568.

BRENNER, M. & PFISTER, R. W. (1951). Enzymatische Peptidsynthese. *Helv. Chim. Acta*, **34**, 2085.

BRENNER, M., SAILER, E. & RUFENACHT, K. (1951). Enzymatische peptidsynthese. *Helv. Chim. Acta*, **34**, 2096.

BRUES, A. M., TRACY, M. & COHN, W. E. (1949). Nucleic acids of rat liver hepatoma: their metabolic turnover in relation to growth. *J. Biol. Chem.*, **155**, 619.

CASPERSSON, T., LANDSTRÖM-HYDÉN, H. & AQUILONIUS, L. (1941). Cytoplasmanukleotide in Eiweissproduzierende Drüsenzelle. *Chromosoma*, **2**, 111.

CHANTRENNE, H. (1950). Synthèses peptidiques à partir d'un dérivé du glycylphosphate. *Biochim. Biophys. Acta*, **4**, 487.

CHANTRENNE, H. (1951). The requirement for coenzyme A in the enzymatic synthesis of hippuric acid. *J. Biol. Chem.*, **189**, 227.

CHANTRENNE, H. & LIPMANN, F. (1950). Coenzyme A dependence and acetyldonor function of the pyruvate-formate exchange system. *J. Biol. Chem.*, **187**, 757.

CHEN, S. Y., EPHRUSSI, B. & HOTTINGUER, H. (1950). Nature génétique des mutants à déficience respiratoire de la souche B-11 de la levure de boulangerie. *Heredity*, **4**, 337.

COHEN, S. S. (1951). The synthesis of nucleic acid by virus infected bacteria. *Bact. Rev.*, **15**, 131.

DAVIDSON, J. N. (1950). *The biochemistry of the nucleic acids*. London: Methuen.

DAVIDSON, J. N., LESLIE, J. & WAYMOUTH, C. (1949). The nucleoprotein content of fibroblasts growing *in vitro*. *Biochem. J.*, **44**, 5.

DOUDOROFF, M., BARKER, H. A. & HASSID, W. Z. (1947). The mechanism of action of phosphorylase as a glucose transferring enzyme. *J. Biol. Chem.*, **168**, 725.

EPHRUSSI, B. & HOTTINGUER, H. (1950). Direct demonstration of the mutagenic action of euflavine on baker's yeast. *Nature*, **166**, 956.

EPHRUSSI, B. & SLONIMSKI, P. (1950). La synthèse adaptative des cytochromes chez la levure de boulangerie. *Biochim. Biophys. Acta*, **6**, 256.

FRIEDRICH-FREKSA, H. (1940). Bei der Chromosomenkonjugation wirksame Kräfte und ihre Bedeutung für die identische Verdopplung von Nucleoproteinen. *Naturwiss.*, **28**, 376.

FRUTON, J. S. (1950). The role of proteolytic enzymes in the biosynthesis of peptide bonds. *Yale J. Biol. Med.*, **22**, 263.

FRUTON, J. S., JOHNSTON, R. B. & FRIED, M. (1951). Elongation of peptide chains in enzyme catalyzed transamidation reactions. *J. Biol. Chem.*, **190**, 39.

GEIGER, E. (1950). The role of the time factor in protein synthesis. *Science*, **111**, 594.

GROSSOWICZ, N., WAINFAN, E., BOREK, E. & WAELSCH, H. (1950). The enzymatic formation of hydroxamic acids from glutamine and asparagine. *J. Biol. Chem.*, **187**, 111.

HAMMARSTEN, E. (1951). Turnover rates during formation of proteins and polynucleotides in regenerating tissues: Isotopes in Biochemistry. A *Ciba Foundation Conference*, p. 103. London: Churchill.

HANES, C. S., HIRD, F. J. & ISHERWOOD, F. A. (1950). Synthesis of peptides in enzymic reactions involving glutathione. *Nature*, **166**, 288.

HAUROWITZ, F. (1949). Biological problems and immunochemistry. *Quart. Rev. Biol.*, **24**, 93.

HAUROWITZ, F. (1950). *Chemistry and Biology of Proteins*, p. 340. Acad. Press, N.Y.

HOKIN, L. E. (1951*a*). The synthesis and secretion of amylase by pigeon pancreas *in vitro*. *Biochem. J.*, **48**, 320.

HOKIN, L. E. (1951*b*). Amino acid requirements of amylase synthesis by pigeon pancreas slices. *Biochem. J.*, **48**, XL.

HOTCHKISS, R. D. (1948). Etudes chimiques sur le facteur transformant du pneumocoque. *Colloques Int. C.N.R.S.* **8**, 57. Paris.

HULTIN, T. (1950). Incorporation *in vivo* of ^{15}N-labelled glycine into liver fractions of newly-hatched chicks. *Exp. Cell. Res.*, **1**, 376.

JEENER, R. (1949). Distribution of *ribosenucleic* acid in the cytoplasm of growing cells studied with phosphorus-32. *Nature*, **163**, 837.

JEENER, R. (1951). Studies on the evolution of nucleoprotein fractions of the cytoplasm during the growth of a culture of *Polytomella coeca*. *Biochim. Biophys. Acta.*, **8**, 125.

JEENER, H. & JEENER, R. (1952). Cytological study of *Thermobacterium acidophilus* R 26 cultured in the absence of desoxyribonucleosides or uracil. *Exp. Cell. Res.* (in the press).

JEENER, R. & SZAFARZ, D. (1950). Relations between the rate of renewal and the intracellular localization of *ribosenucleic* acid. *Arch. Biochem.*, **26**, 54.

JOHNSTON, R. B., MYCEK, M. & FRUTON J. S. (1950*a*). Catalysis of transamidation reactions by proteolytic enzymes. *J. Biol. Chem.*, **185**, 629.

JOHNSON, R. B., MYCEK, M. & FRUTON, J. S. (1950*b*). Catalysis of transpeptidation reactions by chymotrypsin. *J. Biol. Chem.*, **187**, 205.

JORDAN, P. (1944). Zum Problem der Eiweiss Autokatalysen. *Naturwiss.*, **32**, 20.

KELLER, E. B. (1951). Turnover of proteins of cell fractions of adult rat liver in vivo. *Federation Proc.*, **10**, 206.

LEE, N. D., ANDERSON, J. T., MILLER, R. & WILLIAMS, R. H. (1951). Incorporation of labelled cystine into tissue protein and subcellular structure. *J. Biol. Chem.*, **192**, 733.

LE PAGE, G. A. & HEIDELBERGER, C. (1951). Incorporation of glycine-2-C^{14} into proteins and nucleic acids of the rat. *J. Biol. Chem.*, **188**, 593.

LINDERSTRØM-LANG, K. (1947). Synthesis of proteins. *Proc. 6th Int. Cong. Exp. Cytol.* Stockholm.

LIPMANN, F. (1941). Metabolic generation and utilization of phosphate bond energy. *Advances in Enzymology*, **1**, 99.

LIPMANN, F. (1948). Biosynthetic mechanisms. *Harvey Lectures*, **44**, 119. New York: C. C. Thomas.

LIPMANN, F. (1949). Mechanism of peptide bond formation. *Federation Proc.*, **8**, 597.

LONDON, I., SHEMIN, D. & RITTENBERG, D. (1950). Synthesis of heme *in vitro* by the immature non-nucleated mammalian erythrocytes. *J. Biol. Chem.*, **183**, 749.

LURIA, S. E. (1950). Bacteriophage. An essay on virus reproduction. *Science*, **111**, 507.

LURIA, S. E. (1951). Mechanism of bacteriophage reproduction. *Federation Proc.*, **10**, 582.

MONOD, J. (1950). Adaptation, mutation and segregation in the formation of bacterial enzymes. *Biochem. Soc. Symp.*, **4**, 51. Cambridge University Press.

MONOD, J. (1948). Facteurs génétiques et facteurs chimiques spécifiques dans la synthèse des enzymes bactériens. *Colloques Intern. C.N.R.S.*, **8**, 181. *C.N.R.S.* Paris.

OLSON, C. K. & BINKLEY, F. (1950). Enzymatic hydrolysis of cysteinylglycine. *J. Biol. Chem.*, **186**, 731.

PAULING, L. (1940). A theory of the structure and process of formation of antibodies. *J. Amer. Chem. Soc.*, **62**, 2643.

PAULING, L. & COREY, R. B. (1951). The pleated sheet, a new layer configuration of polypeptide chains. *Proc. Nat. Acad. Sci. Wash.*, **37**, 251.

PERUTZ, M. F., LIQUORI, A. M. & EIRICK, F. (1951). X-ray and solubility studies of the haemoglobin of sickle-cell anaemia patients. *Nature*, **167**, 929.

PETERS, T. & ANFINSEN, C. B. (1950). Net production of serum albumin by liver slices. *J. Biol. Chem.*, **186**, 805.

RITTENBERG, D. & SHEMIN, D. (1946). Isotope technique in the study of intermediary metabolism: *Currents in Biochemical Research*, p. 261, N.Y. Interscience.

RONDONI, P. (1940). Über einige fragen der Proteinsynthese. *Enzymologia*, **9**, 380. (1949). Der Aufbau der Eiweisskörper im tierischen Organismus. *Ergeb. Enzymforsch.*, **10**, 65.

SANGER, F. & TUPPY, H. (1951). The amino acid sequence in the phenylalanyl chain of insulin. *Biochem. J.*, **49**, 463.

SCHOENHEIMER, R. (1942). *Dynamic state of the body constituents.* Cambridge, Mass.

SIEKEVITZ, P. & ZAMECNIK, P. C. (1951). *In vitro* incorporation of ^{14}C d-l-alanine into proteins of rat-liver granular fractions. *Federation Proc.*, **10**, 246.

SIMPSON, M. V. (1951). *In vitro* studies on mechanisms involved in protein turnover. *Federation Proc.*, **10**, 247.

SPECK, J. F. (1949). The enzymatic synthesis of glutamine, a reaction utilizing adenosine triphosphate. *J. Biol. Chem.*, **179**, 1405.

SPIEGELMAN, S. (1946). Nuclear and cytoplasmic factors controlling enzymatic constitution. *Cold Spring Harbor Symp.*, **11**, 256.

SPIEGELMAN, S. (1949). Differentiation as the controlled production of unique enzymatic patterns. *Symp. Soc. Exp. Biol.*, **2**, 286. Cambridge University Press.

SPIEGELMAN, S. & KAMEN, M. (1947). Some basic problems in the relation of nucleic acid turnover to protein synthesis. *Cold Spring Harbor Symp.*, **12**, 211.

SPIEGELMAN, S. & NOZAWA, M. (1945). On the inability of intact yeast cells to ferment their carbohydrate reserves. *Arch. Biochem.*, **6**, 303.

SPIEGELMAN, S., SUSSMAN, R. & PINSKA, E. (1950). Cytoplasmic nature of long term adaptation in yeast: *Proc. Nat. Acad. Sci.* U.S.A., **36**, 391.

SPRINSON, D. B. & RITTENBERG, D. (1949). The rate of interaction of the amino acids of the diet with the tissue proteins. *J. Biol. Chem.*, **180**, 715.

STUMPF, P. K. & LOOMIS, W. D. (1950). Observations on a plant amide enzyme system requiring manganese and phosphate. *Arch. Biochem.*, **25**, 451.

SWENSSON, P. A. (1950). The action spectrum of the inhibition of galactozymase production by ultravoilet light. *Proc. Nat. Acad. Sci.* U.S.A., **36**, 699.

SZAFARZ, D. (1951). (Personal communication.)

THORELL, B. (1947). The relation of nucleic acids to the formation and differentiation of cellular proteins. *Cold Spring Harbor Symp.* **12**, 247.

VIRTANEN, A. I. (1950). On nitrogen assimilation and protein synthesis. *Ann. Acad. Sci. Fennicae*, **A. 39**, 1.

WALEY, S. G. & WATSON, J. (1951). Rearrangement of the amino acid residues in peptides by the action of proteolytic enzymes. *Nature*, **167**, 360.

DISCUSSION

L. F. HEWITT: If by a 'template' is meant an exact model of the protein molecule the concept does not appear to be very fruitful since it gives no clue to the mechanism and does not explain why some proteins act as 'templates' and are replicated in the cell whilst others are not. On the other hand if the 'template' is a die or mould forming a counterpart to the protein structure it seems possible that nucleic acids may form such counterparts and function as directors of specific protein synthesis in the cell. Astbury has pointed out, on the basis of X-ray measurements, that the distance apart of the projecting nucleotides in the nucleic acid fibre is about 3·4 Å and this is approximately the distance between amino-acid side-chains in an extended polypeptide chain. Hence, even if the structure were helical, the distribution of the purine and pyrimidine rings in the nucleic acid molecule may result in a specific arrangement of amino acids in juxtaposition with the nucleic acid. The next stage would be the formation of peptide bonds and the linking together of the amino acids in the appropriate order leading to the synthesis of the specific protein molecule. The nucleic acid-protein counterpart theory is in accord with some of the modern work on bacteriophage multiplication dealt with later in the symposium. It also seems possible that the polysaccharide or carbohydrate core present in many proteins may play an important part in the building-up of the protein molecule. Preliminary experiments, however, in the case of serum proteins indicate that the carbohydrate core, consisting probably of galactose, mannose and acetyl-hexosamine may not be of sufficient complexity to control the specific arrangement of amino acids in the protein molecule.

H. CHANTRENNE: A discussion on the advantages of a mould-cast system as compared to a model copying system is to be found in Dr Lwoff's paper in this symposium.

The idea that nucleic acid is part of the template or is the template itself is acquiring much weight indeed; and some recent data reported at this meeting on the respective behaviour of the phage constituents suggests that desoxyribosenucleic acid is probably the important moiety. In this realm of speculation, I would also be inclined to believe that nucleic acid acts as the organizer of the protein, although one has to recognize that no one visualizes very clearly how a nucleic acid can control the formation of a protein pattern.

Now, the general tendency is to consider a template as an exact model or a perfect mould of the corresponding protein. I think this might be too restrictive a view. It is quite conceivable that a template process be involved in organizing part of the protein molecule only, and that once the formation of a part of the protein—say an octopeptide—has been rigidly controlled by a template process, the further development of the molecule might be determined by the structure of the first part and perhaps environmental factors together. For instance, in a helical polypeptide chain, once two turns have been built, the nature of the amino acid which is bound next might depend on the nature of the last amino acid in the part already formed, on that of the amino acids located opposite in the previous turn of the helix, and possibly also on the presence of other molecules in the surroundings. Such a process of epigenesis of the proteins would include the essential idea of a template mechanism, and allow for the interference of various agents with the formation of a protein, as required by enzymatic adaptation or antibody production. This less rigid mechanism would also account for a fact that Dr Pirie has, on several occasions, insisted on, namely that what we used to call a well-defined protein is a mixture of closely related molecular species rather than a pure substance.

The above is intended to emphasize that many different types of mechanisms can be imagined as variations on the theme of a template process.

N. W. PIRIE: Could you amplify your suggestion that after transpeptidization forbidden arrangements may be hydrolysed selectively by specific proteolytic enzymes. The idea is similar to the 'scavenging' process that Bawden and I invoke but I do not know of any good evidence that peptides do differ greatly in the readiness with which they are hydrolyzed.

H. CHANTRENNE: I have not worked with peptidases myself, but several examples of peptidases showing a considerable degree of specificity have been reported; see e.g. the review by E. Smith in *Advances in Enzymology*, Vol. 12. I understand that a high specificity of proteinases and peptidases is the original stimulus behind Bergmann and Fruton's idea that these enzymes play a part, as specific transpeptidases, in protein synthesis. And I find it easier to visualize hydrolytic enzymes as scavengers than as builders, for their efficiency as scavengers is bound to be much higher.

D. J. BAUER: If proteins are synthesized on a template, does this mean that the template is an enzyme? The template is presumably a nucleoprotein, and so far no enzyme activities have been attributed to compounds of this type.

H. CHANTRENNE: This is to a large extent a matter of definition. We don't know of any simple biochemical reaction catalysed by a nucleoprotein, and we also don't know what the catalysts involved in protein synthesis are like. I have used the term enzyme with the general meaning of 'biochemical catalyst'.

VIRUS MULTIPLICATION CONSIDERED AS A FORM OF PROTEIN SYNTHESIS

F. C. BAWDEN & N. W. PIRIE

Rothamsted Experimental Station, Harpenden

The title of the symposium suggests a generalization that may well be unjustified. The only features uniting the virus group, by tacit definition, are that they are smaller than visible organisms and that they have not been cultivated *in vitro*. These two essentially negative features provide no sure grounds for assuming any uniformity in their method of multiplication or other behaviour. There is no obvious reason why the resolving power of ordinary microscopes should have any biological significance, and an apparent inability to multiply except in living hosts is not a feature peculiar to sub-microscopic particles. Present knowledge on the particle sizes of different viruses suggests that they cover a range extending from a little less than that of some bacteria to no more than some proteins which lack virus-like attributes. Within this range there is much opportunity for large differences in chemical constitution, in origins, and in methods of multiplication. Facile generalization, therefore, is to be deplored, and statements that viruses are 'small organisms' should be regarded with as much suspicion as statements that they are 'simply molecules'. Some viruses can be profitably studied by methods developed in other subjects, but all viruses are not amenable to the same methodological approach.

THE ORIGINS OF VIRUSES

In this paper we shall regard an infection as invariably the result of some form of transmission from a previous infection. There is little novelty, however, in the apparently spontaneous appearance of infections, often with viruses the worker is not consciously handling and sometimes with ones not previously recorded. The production of bacteriophages in lysogenic bacteria by such treatments as radiation with ultraviolet light (Lwoff, Siminovitch & Kjelgaard, 1950) may provide analogies when seeking clues to the origins of some of these infections, which now remain unexplained but are generally dismissed as contaminations arising by some unknown method of transmission from another infected host. No doubt this interpretation is usually correct, for viruses probably contaminate hosts far more often than they evolve from them, but it is not one that helps to throw any light on the possible origins of viruses. Transmission from one infected host to another is obviously a process that cannot have continued indefinitely. An initial origin for a virus must be postulated, and some discussion of the possibilities is relevant to our subject, because viruses with different origins are likely also to differ in their methods of reproduction.

Two alternative, but not mutually exclusive, theories have been proposed for the evolution of viruses. The first is that they have arisen from bacteria by a loss of structures and functions which became possible when they adopted an intracellular habitat (Green, 1935; Laidlaw, 1938). Needham (1938) and Lwoff (1943) have examined the principles governing the process whereby autotrophic bacteria can adopt an increasingly parasitic habit and evolve by the successive loss of capacities, so that, for growth, they need media of increasing complexity. The deficiencies developed in this way, though important, will usually affect the synthesis, from even simpler and more widely distributed substances, of small diffusible molecules, such as amino acids. At this stage, the physiology of a bacterium imposes a limit, for an organism bounded by a semi-permeable membrane must be able to synthesize such essential substances as cannot pass through the membrane. To extend this process of degeneration to the stage of viruses, which seem to depend on their environment for at least some of the enzymes required for their own synthesis, it appears necessary to postulate the loss of a surrounding semi-permeable membrane. Free from the restrictions imposed by a membrane, a virus could accrete to itself, from the host cell, particles of any size, and use host-cell enzymes to synthesize components that are incorporated into it. Formally there is no sharp distinction between this type of reproduction and the reproduction of yeast by budding, for various kinds of particles move into developing buds and start processes there. The bud, considered as a new organism, uses parental synthetic systems to make materials of all degrees of complexity until it is sufficiently equipped to function independently.

Although some viruses, particularly some of the larger ones, may have evolved from more independent organisms in such a manner, the smaller plant viruses, with which we shall be concerned in this paper, probably have a different origin. It is the smaller viruses that pose the problem of method of multiplication in its most acute form. At the upper limit of size there is a kind of 'no-man's-land', where virus particles and 'filter-passing' forms of bacteria tend to merge, and some of these types, which were first looked on as viruses, have been grown on artificial media. It is logical, even though not necessarily true, to assume that some large viruses may multiply in ways not entirely dissimilar from the smallest organisms. It is less logical to expect the same processes with the smaller viruses. For these, the theory that derives them by evolution from normal cell components seems more plausible; it stems directly from Muller's (1922) suggestion that viruses might be 'free genes', but there is no reason to postulate one particular source, either nuclear or cytoplasmic, as more likely than another. On this theory, a virus could arise endogenously, because of some accidental change in a normal cell component that prevents it from filling its normal role but not from stimulating the synthesizing machinery of the cell; or it could arise exogenously, by the entry of a normal component from a cell of one organism into a cell of another.

SIMILARITIES BETWEEN PLANT VIRUSES AND OTHER PROTEINS

The plant viruses that have been studied in any detail have been isolated in the form of nucleoprotein preparations, so that the normal nucleoproteins seem the likely place to seek virus origins. Any attempt to generalize, however, is hazardous, for less than 10% of the known viruses have been studied chemically, and amenability to the methods of isolation yet used may depend on a chemical uniformity in this group; this uniformity need not extend to all the other 90%. In what follows we shall use the word virus, unless qualified, exclusively for these small viruses, which are the only ones with which we have worked.

Purified preparations of some of these viruses appear homogeneous when subjected to the customary physico-chemical tests, but this is no reason to assume that they are homogeneous in the sense that every particle is identical with every other and is capable of initiating infection in a susceptible cell. Indeed, there is good evidence with some that this is not so. Nevertheless, the preparations consist of material that is not present in the normal host and, even if the preparations do not consist exclusively of virus particles in the ideal sense, they consist of material whose synthesis is intimately bound up with the synthesis of infective particles.

As these smaller viruses appear to be nucleoproteins, the problem of their multiplication becomes part of the broader problem of nucleoprotein synthesis and of protein synthesis in general. It could be argued that its solution must await the solution of the broader problem, but such an argument misreads the usual history of scientific development. Information gained in the study of a specific problem is often essential for the solution of the general one, and it may well be that the study of virus multiplication will shed more light on protein synthesis than *vice versa*. At present there is little that can be said with certainty about either process, but it is worth comparing virus multiplication with synthesis of proteins in general to see whether there is any phenomenon that is characteristic of this multiplication and is not found elsewhere.

Proteins play so many roles, ranging from the small-scale control exercised by the protein hormones to the large-scale nutritional functions of serum proteins, that it is probably as hazardous to generalize about their mode of synthesis as about virus multiplication. Nevertheless, two main types of response to changes can be distinguished. First, the level of some proteins, e.g. a plasma protein or an enzyme, is maintained approximately constant so that a fall in concentration appears to stimulate synthesis. Secondly, synthesis may be started by the stimulus of an unusual substance, e.g. an antigen or the substrate for an adaptive enzyme (on the plausible assumption that at least some adaptive enzymes are proteins), so that a protein with complementary properties is formed. The distinction between these two may be smaller than appears at first sight. If a cell contains a mechanism capable of making a plasma protein, this may run at an approximately constant rate, provided the supply of substrate is adequate, but there may be no corresponding increase in the total amount of protein because it is continually destroyed at a rate depending on the concentration reached. Such a process may appear to be too inefficient to be probable, but this cannot be assumed until something is known about the efficiency with which a cell synthesizes, destroys and resynthesizes proteins. It is worth stressing that even materials normally regarded as structural or reserve are now known to be maintained by similar dynamic processes. A commonplace of morphogenesis is that it is more difficult to find what stops synthesis when regeneration is complete, or full size attained, than it is to find what started synthesis. Normally proteins meeting certain specifications are produced by the operation of controls into whose nature we need not at the moment enquire. To account for the second type of synthesis we have only to assume that the type of stimulus which ultimately produces ts complement acts by modifying the control so that the normal mechanism now produces modified protein.

There is a vast literature of hypothesis and disputation, much of which would have done credit to a medieval Aristotelian, about the way in which an antigen can be held unrecognizably in the cell so that it can affect all subsequent synthesis. In the absence of any evidence that the stimulus does so persist, it seems wisest not to make the unnecessary assumption. Any mechanism that is capable of producing one type of protein could presumably be modified to produce a related type. The synthesizing mechanism is likely to be as dynamic as the other parts of the cell and, if a part of it undergoes a cycle of resynthesis while contiguous to the stimulus, it is a plausible assumption that it will be permanently modified. The production of antibodies, or of adaptive enzymes, differs most obviously from virus multiplication in that the one produces some sort of complement to the stimulus whereas the other produces something that approximates to being a copy. But a copy can be the complement of a complement. For twenty-five years concepts such as mould and template have been used in this connexion without anyone giving them a satisfactory chemical meaning. Until this has been done and there is at least the outline of a picture of the synthesis of opposites, there is no reason to assume that the synthesis of copies differs from it fundamentally.

It seems clear that virus multiplication either needs a smaller amount of initial stimulus than antibody production, or else it occurs at a different site; if this were not so, animal diseases caused by viruses that are active antigens would presumably be unknown. Virus multiplication also demands a greater host-specificity than antibody production. In this respect the production of adaptive enzymes is a closer analogue, for with these there is both host and agent specificity; that is to say, although some bacteria form adaptive enzymes to some substrates, not all bacteria do, nor to all substrates. At least one enzyme, that converting creatine to creatinine, is formed under the stimulus of either the substrate or the product of the reaction. This is a process that more formally resembles infection, for a bacterium is stimulated by a substance to produce more of that substance. The fact that something is known about the intermediate mechanisms should not obscure the fact that the sequences of events are similar. Another resemblance to infection, is that only fleeting contact with the stimulus may be needed to promote enzyme production. Thus Pollock (1950) has found that *Bacillus cereus* produces no more penicillinase after prolonged than after brief exposure to penicillin. Once the new cycle is started, as with infection, it can proceed without a continual supply of new stimulus.

Adaptation seems often, if not always, to consist in the increase of an enzyme normally present in small amounts rather than to the production of a new enzyme (cf. Lederberg, 1950; Stanier, 1951). This distinction, however, may be more apparent than real. No enzyme preparation is quite specific, perhaps because each contains a group of related substances rather than single entities. If this is the reason, different members of a group are likely to have different relative activities towards different substrates, and adaptation may be the stimulation of one member instead of another. So also with antibody production, modified globulins may be produced by the stimulus of an antigen, but it may be that globulins already occurring in amounts at present undetectable are preferentially increased. The fact that nothing resembling the purified preparations of plant viruses have been isolated from uninfected plants suggests, at first sight, that virus multiplication must be an essentially different process that leads to cells containing entirely new products. This conclusion, however, goes far beyond knowledge. Healthy plants contain the mechanism for synthesizing a variety of nucleoproteins, about which little is known. Certainly they do not contain in quantity any free and soluble proteins that resemble tobacco mosaic or tomato bushy stunt virus, but such proteins could be present at levels comparable to those reached by some other viruses. Without a test as sensitive as infectivity, however, they would be undetectable. There is, indeed, no knowledge as to how closely a virus may resemble a normal host protein, and infection could be the greatly exaggerated production of what may be a variant of a normal protein. A slight modification may free a protein from its customary trammels in a dynamic cycle, so that it is protected from wear and can accumulate sufficiently to be detectable.

THEORIES OF PROTEIN SYNTHESIS

Whatever the protein being synthesized, there are two questions to consider. How are peptide bonds made? How are they made in the correct sequence? Emphasis is often laid on the endergonic character of the process: amino acids→protein. This emphasis on energy gives the problem an attractive simplicity and generality, but what is gained in generality is lost in specificity. That is to say half the problem is discarded. Furthermore, although there is much evidence that when the supply of sources of energy to a tissue fails, protein synthesis fails, there is no reason to think that it fails because energy is lacking at the site of synthesis. Synthesis requires a steady supply of parts to be built into the structure, whatever may be the mechanism of building, and in tissues where poisoning or some other cause stops protein synthesis, a failure in the supply of components may be more important than a failure in the supply of energy. This preoccupation with energy leads to a misuse of the concept of 'high energy phosphates' and to a disregard for the fact that substances have specific properties in addition to the readiness with which they undergo hydrolysis and to the amount of energy released on hydrolysis.

The most obvious mechanism of protein synthesis is reversal of enzymic hydrolysis and the implications of such a mechanism have been examined by many people. If this process takes place directly, the equilibrium is far over on the hydrolytic side and a source of energy is needed to drive it. The amount of energy is indeterminate because in nature the system is not in equilibrium. When synthesis goes on in solution, its products may be removed by absorption on to surfaces or may be sequestrated from back reactions by the formation of complexes; when synthesis goes on at a surface, equilibrium calculations lose their meaning if the products diffuse away. The net result is that improbable reactions can be driven by exergonic reactions not chemically linked with them and these may even be distant processes maintaining osmotic pressure or streaming. Furthermore, even in a closed system the amount of energy needed is not at present determinate for, as Linderstrøm-Lang (1949) has pointed out, the larger the molecule the more likely becomes the addition of another link to it. We may say therefore that, although there is no evidence that synthesis does proceed by a reversal of hydrolysis, many of the arguments that have been advanced to show that it does not are irrelevant.

If protein synthesis starts from amino acids, then it is an improbable process and requires an energy source somewhere to drive it. Some ingenuity has been expended in devising energy sources linked to the synthesis, but in recent years there has been a tendency to move the problem one stage further back and to postulate a source of amino acid derivatives of higher energy content and of such nature that their linking together is an exergonic, and therefore more probable, process. Only the linking together would, on this view, go on at the actual site of synthesis. Among the suggested derivatives we may mention: peptides (Linderstrøm-Lang, 1949), amino-aldehydes (Delbruck, 1941), acetylated amino-acids (Rittenberg & Shemin, 1946), phosphorylated amino acids (Chantrenne, 1950; Lipmann, 1941). This is not the place to discuss the relative probabilities of these substances being the actual pieces from which proteins are built, but we may accept the picture that at the site of synthesis the process may be exergonic, and, with some of the mechanisms proposed, it could even be non-enzymic.

In considerations of the specificity of protein synthesis, and recently of virus synthesis, the old idea of a precursor or 'ogen' has an amazing vitality. It is established that trypsinogen, for example, is turned by trypsin into trypsin, and the principle has been extended to the suggestion that an inactive precursor is turned into virus by an incoming virus inoculum. The evocative idea is clearly irrelevant to virus multiplication; the characteristic feature of the observations with proteolytic enzymes is that the specificity of the final product is controlled by the 'ogen' and not by the stimulus that turns it into enzyme. Thus pig pepsinogen is turned into pig pepsin by pepsin from the chicken (Northrop, Kunitz & Herriott, 1948). With viruses the position is quite different and the infecting agent is the influence that controls the end product. Furthermore the idea explains nothing but an end stage. Suppose an organism is endowed with a battery of 'ogens', each waiting to be converted into a virus by each of the hundred or so viruses to which it may be susceptible, now the synthesis of 'ogens' instead of the synthesis of viruses has to be discussed. Recognizing this the same 'ogen' can be suggested as a precursor for several viruses and as a normal intermediate in the synthesis of some other protein or proteins. By the time this emendation has been made, the idea has lost most of its charm; the specificity of the 'ogen' has disappeared, along with the idea of conversion by a simple transformation. The statement now being made is that all nucleoproteins have similar components and that virus production involves a derangement of protein synthesis or a sequestration of protein intermediates: this is our thesis.

VARIATIONS IN END-PRODUCTS

Protein specificity is an undoubted fact, but in discussing how specificity is attained it is important that it should not be over-rated and mechanisms devised able to achieve greater precision than there is any need to postulate. Thus with no virus is it known how close to identity the amino acid sequence in two particles must be for both to be infective; even if there is a necessary unique sequence, it is unknown what proportion of the particles in any preparation has it. Furthermore, even if all the final particles are identical, this need not be the result of a synthetic mechanism working with absolute precision; a group of related structures may be synthesized in a region that retains only the correct ones and allows the others to be destroyed or to diffuse away; or an immense range of proteins or protein fragments may be made at one site and specific synthesis may consist of the selection from this group of the correct structures. Precision in the final product, whether this is recognized by the apparent homogeneity of certain proteins such as insulin or by the apparent uniformity of genes (cf. Haldane, 1948), may only mean that there is an efficient scavenging mechanism.

Many selection mechanisms have been postulated that could act either to remove specific proteins from a mixture or to select amino acid derivatives as a prelude to arranging them in a defined sequence.

Absorption has already been mentioned as a possible mechanism, acting as an actual rather than hypothetical Maxwell Demon, for driving a reaction by removing a product from an equilibrium system. Specific absorptions are well known and they, by removing only one product out of a mixture, would leave the others to go on round the cycle and so promote specific synthesis. Delbruck (1941) has suggested that a peptide chain would, because of resonance between amino-acid side chains in it and amino-acid derivatives in the ambient solution, build a row of amino-acid derivatives along side of it and in the same sequence. This suggestion resembles Fabergé's (1942) suggestion that chromosome pairing may be due to Guyot-Bjerknes forces. Systems with the same vibration frequency, whether electronic or mechanical, come together because the energy content of a congruent pair is lower than that of any other pair, or than of the separated components, so that if the components approach ,because of some random process such as thermal agitation, they will tend to stay together. If the formation of peptide bonds is a locally exergonic process, the energy evolved can presumably facilitate the release of the copy from juxtaposition with the model. Such an action is therefore autocatalytic; the virus or other model engineers its own synthesis. Some mechanism in the cell may have to hold it still, or straight, or something, and a supply of the connecting pieces has to be maintained, but no other mechanism is needed.

Such a picture offers a ready explanation of the apparent absence of enzymes from the smaller viruses. But this absence should not be taken too seriously. Viruses are certainly free from the types of enzyme that have been sought, but it is unknown as yet how to test for a protein-synthesizing enzyme, supposing there is such a thing. It is not known either what substrate it would work on, or the type of environment in which synthesis might proceed. Until a protein-synthesizing enzyme can be tested for, the apparent absence of enzymes from viruses is without significance, for this is the only type that there is any need to postulate.

It may be argued that vital multiplication, and so virus multiplication, if the idea that viruses are all degenerate micro-organisms should prove to be true, differs from molecular multiplication because the former, although it may produce particles that are morphologically and taxonomically identical, need not produce chemically identical particles. This is true, but it is only relevant if the products of molecular multiplication are known to be identical. The argument that they are not has already been set out (Pirie 1950*b*, 1952); such substances as pepsin and hyaluronic acid are names for categories not identities. This distinction, therefore, arises at the macro-molecular level of complexity and is not deferred till the taxonomic or species level. As soon as a system reaches a certain level of complexity, not all its properties need rigid definition for it to be able to bring about its characteristic activities. This is fully recognized in everyday life; thus although first violins are obviously different, a musical score can be written on the assumption that they are more or less interchangeable; also, lawyers find it useful to draw up schedules of things that may be deemed fungible.

Active though the selective mechanisms may be in determining the character of end products of protein synthesis, it is clear that the individual particles of many proteins are not chemically identical, so that to speak of them as molecules, in the chemists' restrictive sense of the term, may be misleading. This is also true of the products of virus multiplication. There is now much evidence that virus-infected plants do not contain one anomalous protein identical in every respect with the initially infecting particle, but a group of related substances that may differ from one another in various ways. This fact tends to be obscured in the literature, because the main preoccupation of many workers has been to establish the identity of their purified preparations as the viruses themselves, and they have tended to over-emphasize the importance of the apparent homogeneity of some of their carefully fractionated products. Even when, as with some of the virus preparations that can be crystallized, the purified products show no signs of heterogeneity when subjected to customary physico-chemical tests, there is still no reason to assume complete homogeneity. Differences of 1% in particle weight, for example, would not show in sedimentation measurements, and 1% of a tomato bushy stunt virus particle is equivalent to a 'molecular weight' of about 100,000. Such tests, too, can fail to distinguish between infective and non-infective particles, and there is, as yet, no test to tell what proportion of any preparation is infective; all that can be done is to measure the relative infectivity of different preparations, and there is a wide gap between the end-point reached by the most infective preparations and the theoretical end-points of preparations ideally infective. Most of the particles in even the best preparations may be non-infective.

The occurrence of mutants clinically distinct from the strain used as inoculum is, of course, itself evidence that virus multiplication does not lead solely to a precise replication of the initial stimulus, but the heterogeneity of end products is far greater than this. This fact is readily demonstrated with tobacco mosaic virus. In the work published to support the view that the particles are all of one kind, the small particles, which amount to about 30% of total anomalous material in the sap, are ignored, discarded or aggregated. If extracts from infected plants are kept in conditions that minimize aggregation while they are subjected to a series of differential ultracentrifugations, the anomalous nucleoprotein can be separated into a series of fractions with particles of different infectivities, different average length, different physical properties and different serological behaviour, though with the same serological specificity (Bawden & Pirie, 1945; Crook & Sheffield, 1946). The infectivity per unit weight of the different fractions increases with increase in the mean particle length and is maximal with preparations containing predominantly particles about 280 mμ long. There is, however, no reason to assume that all particles of this length are identical or infective. On the contrary, it is very probable that they are not. Many treatments, including heating, exposure to phosphate, or simply to the conditions obtaining in expressed sap, make the small particles aggregate linearly, so that they come to resemble the longer ones in size and physical and serological behaviour; such treatments, however, do not increase infectivity. Similarity of length, therefore, is no guarantee of similar biological activity, nor is it evidence of similar antecedents. The non-infective materials produced during this and other virus infections deserve more attention than they have yet received, for only by a knowledge of the differences between them and infective particles are the phenomena responsible for self-replication likely to become understood. These differences may well be slight, for viruses lose infectivity with many treatments that cause no gross changes in morphology or serological specificity. Although incapable of multiplying, such non-infective material is not devoid of all activity in host cells; for example, after inactivation by ultraviolet irradiation, bacteriophage (Luria & Delbruck, 1942) influenza virus (Henle & Henle, 1943; Henle & Rosenberg, 1949) and tobacco mosaic virus (Bawden & Kleczkowski, 1952*b*) still affect their hosts and interfere with the multiplication of infective virus.

The significance of the short particles that have the serological specificity of tobacco mosaic virus but either lack infectivity or are weakly infective, still remains to be determined. Williams & Steere (1951) have suggested that they are artefacts produced during the making of specimens for electron microscopy, but this cannot explain the serological evidence that they occur in solutions that have never been dried (Bawden & Pirie, 1945). There is no evidence that they occur in infected cells, but they occur in freshly-made extracts, and treatments used for extraction do not appear to disrupt large particles (Bawden & Pirie, 1945; Takahashi & Rawlins, 1948). The fact that crystalline inclusion bodies contain particles 280 mμ long (Wilkins *et al.*, 1950) does not prove that these are the only ones in infected cells; the crystals contain only a small proportion of the total virus in the cells and crystallization itself may be the factor selecting those of a uniform size. There is no evidence that the small particles are immature fore-runners of infective particles, as has been suggested for the serologically active material that precedes the appearance of infective particles in eggs infected with influenza virus (Hoyle, 1948). As infection with tobacco mosaic virus proceeds, non-infective and infective particles both increase. It may well be that the small particles carrying some of the properties of the virus are artefacts derived by the breaking of a fundamental virus particle, but they are found in the least roughly handled preparations and all the extra treatments that have been used bring about aggregation. It is equally possible that they are sub-units for the synthesis of virus particles, partial structures that never become completed, either because they were faulty or perhaps because there were too many of that particular type of part. That they can be assembled into larger particles, morphologically indistinguishable from infective particles, can readily be demonstrated *in vitro*, and their failure to become infective when they are assembled in this manner perhaps indicates that selective mechanisms operate in infected cells to ensure that the correct parts are brought together in the right sequence. There are fascinating possibilities here that will well repay both study and discussion, but before either can profitably proceed, agreement is needed on the basic fact that infective sap contains components which aggregate and change properties readily, and that most of the treatments so far used for virus purification increase the rate of change.

Other rod-shaped or filamentous viruses have been less studied than tobacco mosaic, but there is abundant evidence that all these occur in a wide range of particle lengths and that the particles can aggregate end-to-end (Bawden & Crook, 1947; Kleczkowski & Nixon, 1950; Bawden & Nixon, 1951). There are two obvious mechanisms for the production of rods or filaments, analogies for which are supplied by a factory assembly line and a sausage- or cigarette-making machine. In the first, small parts would come together and the structure would be assembled by the accretion of many individual units; in the second a finished product would be extruded from somewhere, e.g. the cell wall, microsomes or plastids, and the individual particles would be lengths cut off at intervals during the process.

Discussions about the origins of the rod-shaped viruses often centre on the constancy of their length; such constancy, it is claimed, could occur only if they are produced as definite and completed structures and not by a more or less random aggregation. We have suggested that the constancy of length has been exaggerated, but, even if it is a fact, its cogency is difficult to assess. The factors that produce particles with a uniform size in certain polystyrene preparations that have been used as standards in electron micrography, for example, are unknown; also, in the visible range, little is known of the factors that control crystal size. It is to be expected that the probability of a flaw will increase with increasing size, and, if the total of crystallizable material is constant, the surfaces at which crystallization can proceed diminish with increasing size of crystals. Thus, an approximate balance must be struck between opposing tendencies. The length of rod-shaped virus particles may reflect comparable conflicts, the tendency to aggregate being countered by the probability of a flaw and by increasing fragility, so that the final particles represent the best compromise.

Variations in end-products of infection are not restricted to variation in length of rod-shaped particles, but also occur with spherical viruses. Plants infected with the Rothamsted culture of tobacco necrosis virus invariably contain anomalous particles of two kinds, one approximately twice the diameter of the other (Bawden & Pirie, 1950). Both these seem to be nucleoproteins, but their relationships to one another are undetermined. Most of the small particles seem to lack infectivity, because the crystalline preparations consist mainly of these and are much less infective than preparations containing both sizes of particle. So, too, plants infected with turnip yellow mosaic virus contain at least two different kinds of anomalous particle, both of the same size and serologically similar, but chemically different. One is a nucleoprotein, and infectivity is possessed only by this type; the other seems to be the protein moiety minus the nucleic acid (Markham & Smith, 1949). As with tobacco mosaic virus, there is no evidence to show whether the non-infective material is degraded virus or represents different stages in the assembly of particles, but it is clear that virus multiplication does not lead to a uniform end-product identical with the initial inoculum.

The only plant virus that has been intensively studied without there being published evidence of any variability in end products is tomato bushy stunt. Even with this virus, however, the position is not altogether simple. We have unsystematized evidence that, at certain stages of infection, part of the anomalous nucleoprotein is serologically related to the rest but differs from the bulk of the preparation by being precipitable at pH 4, and part by being precipitable along with solanin at pH 8. Each retains this characteristic precipitability after repeated ultracentrifugation. Our conclusion, therefore, is that with no plant virus infection is there satisfactory evidence that only one anomalous macro-molecule is being made.

DISPENSABLE PROTEINS

The ability to multiply, or to stimulate their own synthesis, at first sight seems to separate viruses sharply from other proteins, but the distinction may be less sharp than it seems. An invisible agent that is transmissible and causes a disease is automatically listed as a virus; these defining characters have no taxonomic value and it is important that they should not be given more weight than they deserve. The transmissibility of viruses is normally only demonstrable because symptoms occur, a fact amply illustrated by the many known examples of carriers, in which virus multiplication proceeds unnoticed until transmission is made to some intolerant species. But it may well be that other proteins are as transmissible as viruses, and multiply as readily, once they are introduced into a suitable host. This state of affairs would pass unrecognized unless obvious symptoms are caused; whether transmissible or not, the occurrence of an anomalous protein would remain undetected unless its synthesis accounts for a large part of the protein metabolism of the host, as with Bence-Jones protein. There is nothing approximating to a protein balance sheet for any tissue, and there is no means of knowing whether a proportion of the normal protein of the cell is dispensable. For all that is known to the contrary, some of the so-called normal proteins may be as anomalous as viruses; they may stimulate synthesis in a comparable way and may be produced by the cell either as complements or copies of originally extraneous stimuli. Viruses are seldom deliberately sought, but recognized only when symptoms are so obtrusive that their existence can no longer be neglected. Attention therefore gets focused on extreme manifestations. A rich vein of trivial and transmissible changes is probably awaiting exploration.

There is now much evidence showing the dependence of virus multiplication on the physiological condition of the host and suggesting an intimate merging of the infecting particles with the general metabolism of the host cells. The appearance of components from infecting bacteriophage particles among the materials with small molecular weight in the infected bacteria (Kozloff & Putnam, 1950; Lesley, French, Graham & van Rooyen, 1951), and the failure to recover the initial particles by disrupting the bacteria (Doermann, 1948), suggest that the particles undergo some change while they are simultaneously so affecting the host metabolism that the nucleic acid synthesized changes from being predominantly of one type to another (Cohen, 1949). Similarly, less tobacco mosaic virus can be recovered from inoculated leaves 24 hours after inoculation than immediately after, and if the infecting particles contain P^{32} some of this later occurs in material that is not related to the virus (Bawden & Nixon, 1952). During the latent period, then, there seems to be at least partial degradation of the virus accompanied by a change in the synthetic mechanisms of the cell to forms that produce material with the characters of the inoculum in addition to, or instead of, some of the materials that were produced before. This period is followed by the appearance of new virus particles. If these particles are formed instead of indispensable proteins, then there is a ready explanation for the development of disease symptoms. It is clear, however, that symptoms do not result simply from the total sequestration of amino acids and nucleotides, for tobacco mosaic virus achieves a higher concentration than other plant virus and does not cause by any means the most severe disease. Also, the fact that different strains may multiply to similar extents, but cause diseases of widely different severities, shows that there are specific effects responsible for symptoms. These effects may be secondary to multiplication and indicate that strains of one virus differ in their ability to interfere with host metabolism, or it may mean that different strains are built from different types of sub-unit, and that the host can more readily provide those of one type than another.

If the infecting particles do not produce their initial effects rapidly, it seems that the host cell has some type of scavenging mechanism by which the particles are removed or rendered inactive. For instance, if bean plants are exposed to high concentrations of carbon dioxide (Kalmus & Kassanis, 1944) or to ultraviolet irradiation (Bawden & Kleczkowski, 1952*a*), their condition is altered temporarily so that they resist infection with tobacco necrosis viruses. After a few hours, they regain their original susceptibility when inoculated with fresh virus, but virus particles introduced during the refractory period cause no lesions. Stable viruses such as tobacco mosaic and tobacco necrosis increase rapidly after the latent period and accumulate in large quantities, suggesting that they are largely separated from the general metabolic activities of the host cells. Direct evidence for the view that the formation of tobacco mosaic virus is largely an irreversible process has also been provided by work with N^{15} (Meneghini & Delwicke, 1951), but there is no reason to assume that this is generally true. Most of the less stable viruses reach a low maximum and then either remain constant or decrease, suggesting that they are continually being destroyed and are kept in dynamic equilibrium with other proteins by a repeated synthesis.

So far as we can see, virus synthesis shows no characteristic that is not shown by one at least of the other forms of protein synthesis. The evidence got by the use of isotopes shows that much of the material from which a virus is made is derived from the medium by the host cell after infection (Cohen, 1948). That is to say, the particular element studied is not derived from a precursor already formed in the cell. *A priori* this was to be expected, but it is well to remember that most of this evidence has been got with radioactive phosphorus and the phosphate radicals of a virus may be in more intimate equilibrium with the general phosphate cycles of the cell than are the other parts of the virus.

THE PRODUCTION OF SYMPTOMS

The two extreme concepts of virus multiplication that have been advocated in the past have in common the fact that they demand no new activity by the host as a sequel to infection. The first treats the host as a culture medium in which the parasite multiplies by some such process as growth and fission, and demands only the sort of metabolities on which the quasi-organism is presumed to grow. There is no evidence for this concept and arguments against it have been presented elsewhere (Luria, 1950; Bawden, 1950, 1951; Bawden & Pirie, 1952). The second treats the virus simply as an activator of a precursor or 'ogen', which is a normal component of the host and is converted directly into virus by a slight change. We have already indicated the inadequacy of this concept and need not consider it further. All the intermediate possibilities assign a more active role to the host; the host-cell enzymes are used wholly or in part to engineer the synthesis and the virus acts in a manner covered, or hinted at, by such words as stimulant, starter, model, jig and template. Essentially this concept involves the idea that the introduced virus attaches itself to a mechanism concerned with protein synthesis in the host cell and affects it in such a way that thereafter one of the products synthesized is virus.

In considering the relationships between viruses and normal protein synthesis two facts stand out clearly. The small, stable viruses that occur in high concentration consist for the most part of pentose-containing nucleoprotein, and there is a growing body of evidence that the sites of protein synthesis in the cell are also rich in this nucleic acid (Brachet & Chantrenne, 1951). From this some possible generalizations emerge. If a protein without any nucleic acid prosthetic group is being made at a nucleoprotein surface, there must come a time when it parts company with the surface. Normally the protein comes away free, but the protein moiety of a virus may be that type of protein that retains the nucleic acid more strongly than does the stroma protein. After synthesis, therefore, part of the machinery comes away instead of only the product. Compatible with this idea, though hardly to an extent that would justify calling it evidence, is the fact that in many viruses the union between the protein and nucleic acid is exceptionally strong. Thus nucleic acid, which is presumed to be initially present as nucleoprotein, can be extracted from normal tissues and from isolated microsomes or microsome fragments (Pirie, 1950*a*) by treatments with salt or acid that do not separate it from such viruses as tobacco mosaic, bushy stunt or tobacco necrosis. Such a mechanism gives another possible interpretation of the lack of correlation between the severity of an infection and the amount of anomalous nucleoprotein present in the infected tissue. The specificity of protein synthesis is as likely to result from specific nucleotide arrangements as from any other type of specific structure. Different anomalous proteins would, therefore, take from the host different types of nucleic acid, and if these are of unequal importance in normal cell metabolism, or can be replaced with unequal readiness, the observed differences become explicable.

This picture presupposes that the protein moiety of a virus differs from the normal protein that is being made. We think this probable, although we have already suggested that, for all that is known to the contrary, a virus infection would be an exaggerated synthesis of a normal component. If such a substance, let us say a nucleoprotein, is normally part of an insoluble structure where it is needed and where it remains, its direct effect can operate only in its immediate neighbourhood. It may be that a related substance introduced into the cell could set the anchored substance free. This would be an effect analogous to the pairing of chromosomes during cell division. There, like cleaves unto like, and this is all that is being postulated. The diversion of attractions (polar, hydrogen bond, etc.), which necessarily follows an attraction between free and anchored substances, could suffice to loosen the bonds holding the anchored. If, having been released, more of the substance were reformed at the original site, there would be continuous production of the free material able to stimulate release elsewhere. When production fails, the normal function will no longer be maintained, and this could cause symptoms. Such a picture has something in common with the 'ogen' pictures, for it postulates normal cell components approximating to the viruses, but it differs from their standard forms in that it is unlikely that an extract could be made which, like trypsinogen, could be activated *in vitro* on a detectable scale.

There is obviously no evidence in favour of either of these pictures, but they are compatible with the facts and illustrate how normal mechanisms could be diverted to virus production. The idea that virus multiplication is a derangement of normal nucleoprotein behaviour leads to the postulation of three types of error relevant to this discussion. The aberrant protein may be set free and circulate, but may then be destroyed by other cell systems or be unable to stimulate the protein synthesizing machinery itself. It may carry nucleic acid with it, but be so thoroughly masked by it or other material as to be unable to act as a further stimulant. The nucleoprotein may be both stable and stimulating to further synthesis. It is only this last type of aberration that holds possibilities of being immediately classed as a virus infection. The second possibility however, has an interest, because it may be the state of affairs in bacteria carrying a lysogenic strain of virus, for which either another strain of bacterium, or the intervention of an experimenter, is needed to make material with the generally accepted properties of a virus.

Pictures such as these help to resolve the disagreement that there has been about the nature and development of bacterial viruses. The early disputes centred around the meaningless question of whether or not they were 'particulate', which is still sympathetically considered in some modern texts (e.g. Topley & Wilson, 1946). A more significant question was their endogenous or exogenous origin. The exogenous view has prevailed, with Felix (1947) one of the few remaining dissenters, but it has done so to some extent by neglecting the evidence that has been obtained for the presence in some bacterial and other viruses of components specific to the host in which the viruses multiply (Knight, 1946; Pirie, 1949). Such differences in composition, and variations of the kind we have described in end products from infections with some plant viruses, could not occur were virus particles as homogeneous as is suggested by the statements of chemists who examine virus preparations predominantly by physico-chemical techniques. It is largely because some biologists believe these extreme claims that they argue unnecessarily in terms of the distinction between rigidly definable chemical substances and the presumably more plastic quasi-organisms. For this reason, it is important to emphasize the irrelevance of most chemical and physical measurements in discussions of the biological activity of large particles. In our opinion, viruses are probably as uniform as most other biologically produced particles of comparable sizes, and in their hosts they are probably produced by the same synthetic mechanisms that produce other nucleoproteins: to this extent, therefore, they can be regarded as endogenous. We see no incompatibility between such properties and the fact that the synthesis of viruses can be promoted by exogenous stimuli.

In this review we have related our argument to protein synthesis, and we conclude that, at least with the smaller viruses, nothing has so far been discovered that necessarily separates virus multiplication from the synthesis of other proteins. This argument, however, accounts for only part of the virus particle. With as much, or as little, justification, we could also have argued that nothing is known that sharply distinguishes virus multiplication from the synthesis of nucleic acid. We chose protein synthesis, because, although ideas about it are still nebulous, they have attained more form than ideas about nucleic acid synthesis. Only recently has it been generally recognized that variations in nucleotide sequence permit extensive isomerism of nucleic acids: not perhaps the gratuitous isomerism that protein structure theoretically permits, but still amply sufficient to cover the amount of specificity for which there is evidence. We might, indeed, with more aptness, have considered the whole problem in terms of the synthesis of nucleic acid, because transmissible nucleic acids are known. This approach would have led to much the same conclusions. Cells synthesize many different types of substance, and each synthesis has its peculiarities. When balance is upset by virus synthesis, some peculiarities obtrude themselves more than others, but there is no evidence that any new type of mechanism must be invoked.

REFERENCES

BAWDEN, F. C. (1950). *Plant viruses and virus diseases.* Waltham, Chronica Botanica.

BAWDEN, F. C. (1951). The multiplication of viruses. *Sci. Prog.*, **153**, 1.

BAWDEN, F. C. & CROOK, E. M. (1947). Some properties of potato virus X in leaf extracts made in different ways. *Brit. J. Exp. Path.*, **28**, 403.

BAWDEN, F.C. & KLECZKOWSKI, A. (1952*a*). Ultraviolet injury to higher plants counteracted by visible light. *Nature*, **169**, 90.

BAWDEN, F. C. & KLECZKOWSKI, A. (1952*b*). Unpublished.

BAWDEN, F. C. & NIXON, H. L. (1951). The application of electron microscopy to the study of plant viruses in unpurified plant extracts. *J. Gen. Microbiol.*, **5**, 104.

BAWDEN, F. C. & NIXON, H. L. (1952). Unpublished.

BAWDEN, F. C. & PIRIE, N. W. (1945). The separation and properties of tobacco mosaic virus in different states of aggregation. *Brit. J. Exp. Path.*, **26**, 294.

BAWDEN, F. C. & PIRIE, N. W. (1950). Some factors affecting the activation of virus preparations made from tobacco leaves infected with a tobacco necrosis virus. *J. Gen. Microbiol.* **4**, 464.

BAWDEN, F. C. & PIRIE, N. W. (1952). Physiology of virus diseases. *Ann. Rev. Plant Physiol.* (in the Press).

BRACHET, J. & CHANTRENNE, H. (1951). Protein synthesis in nucleated and non-nucleated halves of *Acetabularia mediterranea* studied with carbon-14 dioxide. *Nature*, **168**, 950.

CHANTRENNE, H. (1950). Synthèses peptidiques a partir d'un dérivé du glycylphosphate. *Biochem. Biophys. Acta*, **4**, 484.

COHEN, S. S. (1948). The synthesis of bacterial viruses. II. The origin of the phosphorus found in the desoxyribonucleic acids of the T_2 and T_4 bacteriophages. *J. Biol. Chem.*, **174**, 295.

COHEN, S. S. (1949). Growth requirements of bacterial viruses. *Bact. Rev.*, **13**, 1.

CROOK, E. M. & SHEFFIELD, F. M. L. (1946). Electron microscopy of viruses. I. State of aggregation of tobacco mosaic virus. *Brit. J. Exp. Path.*, **27**, 328.

DELBRÜCK, M. (1941). A theory of autocatalytic synthesis of polypeptides and its application to the problem of chromosome reproduction. *Symp. Quart. Biol.*, **9**, 122.

DOERMANN, A. H. (1948). Intracellular growth of bacteriophage. *Carnegie Inst. Wash. Yearbook*, **47**, 176.

FABERGÉ, A. C. (1942). Homologous chromosome pairing: the physical problem. *J. Genet.*, **43**, 121.

FELIX, A. (1947). The nature of bacteriophages. *Proc. 4th Int. Cong. Microbiol.*, p. 355.

GREEN, R. G. (1935). On the nature of filterable viruses. *Science*, **82**, 443.

HALDANE, J. B. S. (1948). The formal genetics of man. *Proc. Roy. Soc. B.*, **135**, 147.

HENLE, W. & HENLE, G. (1943). Interference of inactive virus with the propagation of virus of influenza. *Science*, **98**, 87.

HENLE, W. & ROSENBERG, E. B. (1949). One-step growth curves of various strains of influenza A and B viruses and their inhibition by inactivated virus of the homologous type. *J. Exp. Med.*, **89**, 279.

HOYLE, L. (1948). The growth cycle of influenza virus A. A study of the relations between virus, soluble antigen and host cell in fertile eggs inoculated with influenza virus. *Brit. J. Exp. Path.*, **29**, 390.

KALMUS, H. & KASSANIS, B. (1944). Reduction by carbon dioxide of susceptibility of beans to tobacco necrosis viruses. *Nature*, **154**, 641.

KLECZKOWSKI, A. & NIXON, H. L. (1950). An electron-microscope study of potato virus X in different states of aggregation. *J. Gen. Microbiol.*, **4**, 220.

KNIGHT, C. A. (1946). Precipitin reactions of highly purified influenza viruses and related materials. *J. Exp. Med.*, **83**, 281.

KOZLOFF, L. M. & PUTNAM, F. W. (1950). Biochemical studies of virus reproduction. III. The original virus phosphorus in the *Escherichia Coli* T_6 bacteriophage system. *J. Biol. Chem.*, **182**, 229.

LAIDLAW, P. P. (1938). *Virus Diseases and Viruses.* The Rede Lecture. Cambridge University Press.

LEDERBERG, J. (1950). The Beta-d-galactosidase of *Escherichia Coli*, strain K-12′. *J. Bact.*, **60**, 381.

LESLEY, S. M., FRENCH, R. C., GRAHAM, A. F. & VAN ROOYEN, C. E. (1951). Studies on the relationship between virus and host cell. II. The breakdown of T_{2r} and bacteriophage upon infection of its host *Escherichia Coli. Canadian J. Med. Sci.*, **29**, 128.

LINDERSTRØM-LANG, K. (1949). Synthesis of proteins. *Exp. Cell. Res. Suppl.* **1**, 1.

LIPMANN, F. (1941). Metabolic generation and utilization of phosphate bond energy. *Adv. Enzymology*, **1**, 99.

LURIA, S. E. (1950). Bacteriophage: an essay on virus reproduction. *Science*, **111**, 507.

LURIA, S. E. & DELBRÜCK, M. (1942). Interference between inactivated bacterial virus and active virus of the same strain and of a different strain. *Arch. Biochem.*, **1**, 207.

LWOFF, A. (1943). *L'évolution physiologique: étude des pertes de fonctions chez les micro-organismes.* Herman et Cie, Paris.

LWOFF, A., SIMINOVITCH, L. & KJELGAARD, N. (1950). Induction de production de phage dans une bactérie lysogène. *Ann. Inst. Pasteur*, **79**, 815.

MARKHAM, R. & SMITH, K. M. (1949). Studies on the virus of turnip yellow mosaic. *Parasitology*, **39**, 330.

MENEGHINI, M. & DELWICKE, C. C. (1951). The multiplication of tobacco mosaic virus in the host tobacco plant. *J. Biol. Chem.*, **189,** 177.

MULLER, H. J. (1922). The relation between the genes and the characters of the organism. *Amer. Nat.*, **56,** 32.

NEEDHAM, J. (1938). Contributions of chemical physiology to the problem of reversibility in evolution. *Biol. Rev.*, **13,** 225.

NORTHROP, J. H., KUNITZ, M. & HERRIOTT, R. M. (1948). *Crystalline enzymes.* New York: Columbia University Press.

PIRIE, N. W. (1949). The association of viruses with other materials in the cell and in extracts. *Proc. 6th Int. Cong. Exp. Cytol.*, p. 183.

PIRIE, N. W. (1950*a*). The isolation from normal tobacco leaves of nucleoprotein with some similarity to plant viruses. *Biochem. J.*, **47,** 614.

PIRIE, N. W. (1950*b*). A biochemical approach to viruses. *Nature*, **166,** 495.

PIRIE, N. W. (1952). Concepts out of context: the pied pipers of science. *Brit. J. Philos. Sci.*, **2,** 269.

POLLOCK, M. R. (1950). Penicillinase adaptation in *B. Cereus*: adaptive enzyme formation in the absence of free substrate. *Brit. J. Exp. Path.*, **31,** 739.

RITTENBERG, D. & SHEMIN, D. (1946). The metabolism of proteins and amino acids. *Ann. Rev. Biochem.*, **15,** 247.

STANIER, R. Y. (1951). Enzymic adaptation in bacteria. *Ann. Rev. Microbiol.*, **5,** 35.

TAKAHASHI, W. N. & RAWLINS, T. E. (1948). An electron microscope study of tobacco mosaic virus extracted from pulp and juice after various periods of infection. *Phytopathology*, **38,** 279.

TOPLEY, W. W. C. & WILSON, G. S. (1946). *Principles of bacteriology and immunity.* 3rd edition, p. 329. London: Arnold.

WILKINS, M. H. F., STOKES, A. R., SEEDS, W. E. & OSTER, G. (1950). Tobacco mosaic virus crystals and three-dimensional microscopic vision. *Nature*, **166,** 127.

WILLIAMS, R. C. & STEERE, R. L. (1951). Electron microscopic observations on the unit of length of the particles of tobacco mosaic virus. *J. Amer. Chem. Soc.*, **73,** 2057.

DISCUSSION

C. H. ANDREWES, as Chairman of the Session: Dr Bawden is one of those queer people who are not sure that viruses are organisms. However, his saving grace is that he is sure they are not molecules. In fact, he and Pirie are sitting very nicely on the fence, having built up there a strongly entrenched position. All the same I am sure many people will like to attack them, and I invite them to ‘have a go’.

F. C. BAWDEN: I cannot imagine how a strongly entrenched position can be built up while sitting on a fence, so I don’t know whether this describes our position or not. I doubt it, because I am by no means sure that there is anything equivalent to a fence on which to sit, for this would mean that a sharp division can be made separating one biological type from another. There are obviously some factors linking all those pathogens that we chose to call viruses, for otherwise we would not give them the same name. There is, however, no reason to assume that the whole group is uniform in origin, structure, or method of multiplication. Should there be sufficient uniformity to justify our current grouping, there is still no reason to assume that words such as ‘organisms’ and ‘molecules’, which are borrowed from other scientific disciplines, can be applied to viruses without introducing misleading concepts.

R. C. BACKUS: Dr Bawden's claims of advantage for those engaged in plant virus studies should be qualified by one great disadvantage. This is the disadvantage of a very low ratio of infectivity to quantity of inoculum when attempting to conduct quantitative evaluations.

F. C. BAWDEN: For the health of plants it is fortunate that all uninjured plants appear to be immune from viruses, but I agree that it is a great disadvantage for experimental work. The need to make wounds as a prelude to infection complicates interpretations of infectivity tests and of work on factors affecting resistance to infection. The local-lesion technique is very valuable for measuring the relative infectivities of different virus preparations, but we have no method of finding the absolute infectivity of any one preparation. The low ratio of number of lesions to number of particles found in work with plant viruses can be interpreted differently. It may be that most of the particles, even in the most highly infective preparations, are non-infective, but it is also possible that most are wasted on the surfaces of leaves during inoculation, and never meet the essential entry points.

G. S. STENT: It should, in principle, be possible to test by means of radio-active tracers whether any type of particle appearing within infected cells can qualify as a precursor of mature virus. If *for a short time* during the course of infection the normal substrate of the host is replaced by one containing suitably labelled compounds, then radio-activity should appear in precursor substances *before* it appears in virus particles. At later stages of virus production, the radio-activity should disappear again from the presumed precursor fraction as this achieves maturation as infective units and as new precursors are again formed from non-labelled substrates.

F. C. BAWDEN: I agree that, in principle, radio-active isotopes ought to be used in differentiating between successive stages in the synthesis of virus particles. We thought they might show the origin of and relationship between the various products obtained in extracts from plants infected with tobacco mosaic virus, and we made some experiments in which infected plants were given P^{32} at different times after infection. We then separated the material with the serological specificity of the virus, from both sap and extracts from the fibrous residues of leaves, into small and large particles. We got no conclusive results, for in different experiments there were no constant differences between the proportions of radio-activity in the different fractions. There was no evidence that the small particles were 'precursors' of the large ones or that they were produced as fragments from them.

M. H. SALAMAN: Dr Bawden has said that viruses can be distinguished from other nucleo-protein particles produced in the cell only by the property of infectivity. It seems to be that this property must be the expression of a metabolism fundamentally different from that of other products of the cell. Many viruses continue to multiply until irreparable damage is done to the cell. The increase of all other cellular constituents is related to the health, growth, and reproduction of the cell. I agree with Dr Markham that the fact of infectivity indicates an abnormality, if not in the mechanism of synthesis, certainly in its control in relation to other cellular processes.

F. C. BAWDEN: I hope I did not say that infectivity was the only property that separates viruses from other nucleo-proteins, for it is not. Indeed, pathogenicity is the prime difference, for without this infectivity would not usually be demonstrable. Work with paracrinkle virus, which occurs in all King Edward potatoes without causing a disease, provides a good illustration of this principle. It can readily be transmitted to tomato plants, but this for long remained unrecognized as these also show no symptoms; their infection can be detected only by further transmissions to some intolerant plant or by using electron microscopy to demonstrate the virus particles in extracts from them. Many other particles may also be infective, but this becomes obvious only when they are transmitted to a host in which they cause some abnormality: we then call the particle a virus. There is no fundamental need for a correlation between infectivity (or transmissibility) and pathogenicity; there is an apparent need set by the practical difficulties of recognizing one without the other.

There are undoubtedly mechanisms that control the extent to which many viruses multiply, and there is no reason to assume they differ from mechanisms that control other nucleoprotein production. They must operate with all viruses that do not cause lethal diseases, and only a minority do so. A virus has a strictly limited future in cells without mechanisms that restrict its multiplication to levels below those at which the cells die. It is perhaps misleading that so large a proportion of the work on virus multiplication has been done with bacteriophages and other virus-host combinations that result in the death of the host.

J. D. WATSON: Both the work of Drs Luria and Hershey with bacterial viruses and that of Dr Markham with turnip yellow mosaic virus suggests that the nucleic acid of some virus particles is located in the interior of the particle and is surrounded on the outside by the protein component. I wonder whether we might explain the limitation of infectivity in tobacco mosaic virus preparations to those particles of 2800 Å length by postulating a similar structural plan for this virus. It has always seemed strange that infectivity should be limited to the particles of 2800 Å length if the infective particle consisted of a lengthwise repetition of a number of equivalent sub-units. We should remember that the nucleic acid component amounts to only 6% of the total particle mass and so will probably have only a slight influence on the X-ray diffraction pattern. It is thus possible that only the protein component repeats its pattern every 68 Å and that the nucleic acid component consists of unbroken polynucleotide chains 2800 Å in length, a distance too long to be detected by X-Ray diffraction techniques. That is, the frequent occurrence of particles 2800 Å long may be the consequence of the aggregation of the virus protein around nucleic acid chains of this length.

Since, as first pointed out by Bernal & Fankuchen (*J. Gen. Physiol.*, 1941, **25,** 111), each TMV particle behaves to X-rays like a miniature crystal, it may be profitable to regard the final aggregation of the virus protein as a crystallization process. The formation of a virus particle may therefore follow the same rules which apply to the formation of crystals from smaller monomeric units and the conclusions of Burton, Cabreara, and Frank (*Phil. Trans. Roy. Soc.* A, 1951, **243,** 299) on the mechanism of crystal growth should be considered. These authors conclude from theoretical calculations on the rates of crystal growth, that crystals will grow at a much faster rate if they do not form a perfect lattice structure but instead contain screw dislocations. The presence of screw dislocations increases the rate of crystal growth by permitting the perpetuation of corners into which the monomeric units can fit and thus allows crystal growth to occur at a constant rate, uninterrupted by the formation of closed surfaces. Thus, if TMV rods are built up by a crystallization of protein molecules around a nucleic acid core, there will be a selective advantage for the protein molecules to be helically arranged around the longitudinal axis.

N. W. PIRIE: The published evidence about the infectivity of TMV particles of differing length shows that preparations are more infective as the proportion of 300 mμ particles in them increases. There is no evidence that it is only these particles that carry infectivity; we incline to the view that shorter particles may also cause infection but less readily than the long ones. The reason for suggesting that the nucleic acid is inside a protein tube does not therefore seem to be very well founded. It is, however, by no means excluded in spite of the evidence that Butenandt *et al.* (*J. Physiol. Chem.* 1942, **274,** 276) have presented for the opposite arrangement. I am unwilling to take sides in the controversy though I have pointed out (Pirie, *Ann. Rev. Biochem.* 1946, **15,** 573) that the arrangement of nucleic acid outside protein is in agreement with some of the evidence of chromosome structure and could explain the regularity with which spherical plant viruses have been found to have higher nucleic acid contents than those that can aggregate into rods. Probably each extreme view is an oversimplification and the two components interweave in a manner that may well be different in different viruses.

We have all used analogies from crystallization at various times in interpreting aspects of virus multiplication but their limitations must be emphasized. Typically, crystallization is reversible; if, for example, the solution is warmed, material goes into solution and crystallizes again on cooling. The energy changes are small and co-valent bonds are not formed. There is no evidence that the pieces from which a virus particle is made are held together in this loose way and in the particular example of TMV I would point out that the unit that Watson refers to as a monomer is very much smaller than the particles whose existence Bawden and I have had so much difficulty in getting other workers to accept.

T. H. THUNG: In discussing infectivity and virus multiplication the condition of the host cell as well as that if the virus particles should be considered. The susceptibility of the host can be influenced by many factors, and there is a close interaction between virus and host. The importance of the host has not been stressed enough; whether or not a virus preparation is found to be infective can often depend on the physiological state of the host when inoculated.

F. C. BAWDEN: As Professor Thung knows, much of our recent work at Rothamsted has dealt with the effects of environmental changes on the susceptibility of plants to infection, and on the multiplication of viruses in infected plants. Our results fully support his views of the great importance of the physiological state of the host plant when inoculated: it can determine whether or not infection is established and, if so, the extent to which the virus multiplies, and the type of symptoms evoked.

METABOLIC ASPECTS OF VIRUS MULTIPLICATION

D. J. BAUER

Wellcome Laboratories of Tropical Medicine, London

INTRODUCTION

The metabolism of an organism is usually a fruitful field for research, but has been largely neglected until recently in the case of viruses on account of the special difficulties of the subject and the discouraging results of earlier work. The study of virus metabolism is of special interest, however, in that it affords evidence of the method by which viruses multiply, a question which is still much in dispute.

Before reviewing the historical aspects of the subject it should be pointed out that rickettsiae and bacteriophage are excluded from consideration; the status of the former is not settled beyond doubt, and bacteriophage, although undoubtedly a virus, will be dealt with in other contributions to this symposium; the present review is moreover confined to a consideration of viruses affecting animals and man, and does not include those responsible for insect and plant diseases. The subject of chemotherapy as a whole is also excluded from consideration, since although it has a direct bearing upon virus metabolism the results so far obtained are not helpful to the problem under discussion.

Two periods may be distinguished in research on virus metabolism. During the first period, from 1926 to 1942, viruses, by obvious analogy with bacteria, were thought to have a metabolism of their own, and a considerable amount of work was devoted to the detection of respiratory and other metabolic activities in preparations of virus bodies. This work was mostly carried out with vaccinia, the virus which at that time was most easily obtained in a purified condition. The results were almost entirely negative; the elementary bodies appeared to have no respiration and scarcely any enzyme activity *in vitro*, and that enzyme activity which was actually detected was suspected to be an artefact due to contamination with enzymes present in the tissue from which the virus was derived. The opinion thus arose that viruses, while being nevertheless organisms and in no way inanimate bodies, had no metabolism of their own and thus represented the ultimate stage of parasitism, in which all life activities were borrowed from the host. This opinion still holds at the present time, although there are now firm grounds for considering that a virus is in no way a parasite, at least in the ordinary sense of microbiological usage, and consequently the fact that all its life activities are those of the host should no longer be taken as evidence of extreme degeneration or specialization, but rather as evidence that the viruses are of totally different nature and possibly derive their origin from some constituent of the nucleus or cytoplasm which has developed autonomy.

At a later stage methods of purifying influenza virus became available, using in allantoic fluid a starting material which is itself poor in enzymes and which should thus give unequivocal results, but interest in the subject appears to have waned, since no detailed examination of the metabolic activities of purified influenza virus has so far been reported. In the second period of research, from 1942 until the present time, the centre of interest shifted from the virus to the metabolism of the infected tissue. The economic importance of viruses has caused a concentration of investigative effort on problems of isolation, diagnosis and epidemiology, and the study of the metabolism of infected tissues began slowly and is only now gathering momentum, but it has so far been prolific of results of a type which are of particular relevance to a discussion of the method of multiplication of viruses and also of their fundamental nature. During the same period information has been accumulating in an independent field which nevertheless has a direct bearing upon the present problem, namely the effect of age and nutritional state upon the rate of growth of virus in the experimental animal. It has been found that virus growth is greatly influenced by these factors, and it appears that cell metabolism must be functionally intact for maximal multiplication of virus to take place.

In the following pages these various aspects of the subject of virus metabolism will be reviewed at length, and their bearing upon the problem of virus multiplication will be examined in the concluding section.

ENZYMIC ACTIVITY OF VIRUSES

Work in this field has necessarily been limited at all stages by the degree of development of methods of virus purification on the one hand and the extent of biochemical knowledge on the other. The early work of Bronfenbrenner & Reichert (1926) is crude by present standards and does not contribute any reliable information. These authors attempted to detect respiratory exchanges in brain tissue infected with the viruses of herpes and rabies. The tissue had been kept in glycerine for several months previously in order to eliminate the respiration of the tissue itself. The infected tissues showed no difference in oxygen uptake and carbon dioxide output from normal brain tissue which had been treated similarly, and thus no respiration attributable to the virus could be detected. The concept of titre was probably not fully understood at this time, and the preparations which were used probably contained only small amounts of active virus; no respiratory supplements were added and the negative findings, although not conflicting with subsequent work, can thus have no significance.

In 1935 attention was diverted to vaccinia virus by Breinl and Glowazky. Fresh suspensions of elementary bodies were prepared from infected rabbit testicle by low and high-speed centrifugation. They contained virus to a titre of 10^6 when tested on rabbit skin; this figure is low and the conditions of the experiment were therefore not favourable for detecting enzyme activity in the virus particles. The suspensions were placed in the Warburg apparatus with Ringer containing 0·2% glucose. A considerable uptake of oxygen and liberation of carbon dioxide under aerobic and anaerobic conditions was observed, but this was complete within the first 10 minutes of observation. The authors concluded from this that the elementary bodies carried out metabolic activities and were therefore living organisms. Events occurring within the first few minutes of a respiration experiment are of little significance, however, and the gaseous exchanges observed were probably due to purely chemical processes taking place in oxidizable impurities derived from the tissue of origin.

Methods of purification of vaccinia were now developed further and highly purified suspensions of elementary bodies became available for investigation; vaccinia proved such a suitable test object that work for the next five years was entirely devoted to it. Parker & Smythe (1937) were the first to take up the subject seriously. The suspensions of elementary bodies which they used had been washed repeatedly and contained virus up to a titre of 10^9. A quantity of virus equivalent to a dry weight of 24 mg. showed only a very slight uptake of oxygen in the Warburg apparatus in one hour; the addition of glucose, glucose monophosphate or methylene blue did not increase the consumption of oxygen. The uptake was in any case very much less than that of resting bacteria and ceased after one hour, whereas the virus was still infective at the end of the experiment. A small amount of carbon dioxide was given off by the elementary body suspension, again mostly within the first hour, and the amount was not increased by adding glucose, glucose phosphate or a 'respiratory supplement' in the form of fresh rabbit kidney. The authors also considered the possibility that the virus might contain co-enzymes instead of complete enzyme systems, but addition of virus to suspensions of rabbit erythrocytes did not increase the oxygen uptake of the latter.

It was concluded that no evidence of metabolic activity had been obtained. The experiments were carried out with fresh virus in adequate amounts, and afford valid evidence of the absence of respiration.

MacFarlane & Salaman (1938) extended the field of investigation to certain specific enzyme activities. They found that the elementary bodies of vaccinia lacked dehydrogenase activity towards glucose, hexose monophosphate, α-glycerophosphate, succinate, lactate, malate and certain other substrates when tested by the Thunberg technique with methylene blue or 2:6-dichlorophenol-indophenol. Phosphatase was apparently present, however, since a considerable liberation of phosphate took place when elementary bodies were incubated with β-glycerophosphate for one hour at 37° in the presence of magnesium ions at pH 9. Catalase activity could also be easily detected. The tissue of origin (rabbit skin) of the elementary bodies contained phosphatase and catalase, and the authors considered that 'the possibility that these enzymes are adsorbed on the virus particles cannot be dismissed', although they inclined to the view that the enzymes were an integral part of the virus. The skin also contained dehydrogenases, which were absent from the elementary body suspensions, and this was considered to be additional evidence that phosphatase and catalase were contained in the virus. In a more careful search for dehydrogenase activity MacFarlane & Dolby (1940) added various co-factors (co-enzymes 1 and 2, Warburg's yellow enzyme), but could still find no dehydrogenase activity towards triosephosphate, lactate and hexosemonophosphate in experiments in which about 1·6 mg. of virus were used. Negative results were also obtained with tobacco mosaic and bushy stunt viruses. No zymohexase or enolase activity could be detected in 2·2 mg. of virus, whereas the enzymes were present in the tissues from which the virus had been extracted. There was no evidence of carbohydrase activity, as no sugar was liberated from α-methylglucoside, maltose, cellobiose, lactose, sucrose or adenosine on incubation for 24 hours at 37°, and there was no apparent peptidase action with *dl*-leucylglycine as substrate. Phosphodiesterase was apparently present, however; 2 mg. of virus liberated 0·036 mgm. of phenol from potassium diphenylphosphate in a total volume of 3·75 ml. in 24 hours, and 0·095 mg. in 70 hours. These times of incubation are very long, and no control was done with killed virus. Special precautions were taken to avoid the growth of bacteria and chloroform was added to the suspensions before they were incubated, but the enzyme activities found must still be regarded with suspicion as they may have been derived from the tissue of origin or from bacterial contamination during the purification of the elementary body suspensions. Ribonucleic acid was hydrolysed by 2 mg. of virus, but only after incubation a 37° for from 22 to 99 hours; this was probably due to residual enzym since ordinary tissue extracts or bacterial suspensions will produc measurable hydrolysis after an hour or so. Thymus nucleic acid wa broken down to a slight extent; the substrate used would presumabl have been an impure mixture of oligonucleotides and other breakdow products which might be split non-specifically by phosphatases, and no the highly polymerized acid which is the specific substrate for deoxyribonuclease. A considerable degree of phosphomonoesterase activity wa present; adenosine-5-phosphate and β-glycerophosphate were split to considerable extent when incubated with elementary bodies for tw hours at 37°. Adenosine-3-phosphate was split to a lesser extent. Th authors considered that phosphatases must be an integral part of th virus body, and suggested that the action on adenosine-5-phosphat might provide the virus with a means of regulating carbohydrate metabolism.

Doubt was cast upon the reliability of this assumption by the work o Hoagland and his collaborators (1940, 1941, 1942), who carried out detailed examination of the biochemical activities of purified suspensions of vaccinia elementary bodies. These authors confirmed the absenc of dehydrogenases, and the presence of phosphatases, but went on t show that elementary bodies of known phosphatase activity would adsorb further quantities of the enzyme when they were suspended in dilute solution of pancreatic phosphatase and then removed by centrifugation. The catalase activity of elementary body suspensions was als confirmed, but it was similarly shown that they would adsorb amounts o catalase from solution which were much too large to measure. All of fiv suspensions which were tested hydrolysed tributyrin under anaerobi conditions and thus contained lipase, but it was again found that added enzyme was adsorbed from solution by the elementary bodies. Thes results show conclusively that vaccinia virus has a high affinity fo enzymes and thus presumably adsorbs them from the tissue of origin during the process of purification. It cannot therefore be concluded tha the enzyme activity of a virus suspension resides in the virus particle themselves, and the positive results reported hitherto must therefor be entirely discounted. Hoagland and his collaborators also found tha purified vaccinia virus contained biotin, a flavine and copper. The possible significance of these observations will be discussed later.

The centre of investigation might have shifted at this stage to influenza virus, for which the superior method of purification by red cell adsorption and elution was soon to be developed. The possibility of the repetition of the negative or at best inconclusive findings with vaccinia may have seemed unattractive to most workers, for no planned investigation of the enzyme activities of influenza virus has so far been reported. The subject has a direct bearing upon the theoretical considerations developed later in this review, and should be worth investigating, especially in view of the simplicity of the experimental methods involved. A few personal observations in this field are now described, which are representative of the results obtained in work so far unpublished. The enzyme activity of influenza virus can be tested very simply by observing the enzyme activity of allantoic fluid containing the virus. This is present in high titre (10^9) and comparatively large amounts (10 mg. of virus in 100 ml. of allantoic fluid; Stanley, 1945) and if the infected allantoic fluid lacks a given enzyme activity this is probably missing from the virus particles themselves. If on the other hand a particular enzyme activity is present in the fluid the virus can be specifically removed by red cell adsorption and the presence or absence of the enzyme on the virus can be inferred from a redetermination of the enzyme activity of the fluid after adsorption. All experiments were carried out with the PR8 strain of influenza virus. Four samples of fluid with a titre of 8,000 by the Salk test were examined for xanthine oxidase activity: 2 ml. of fluid, 0·5 ml. of pH 9 borate buffer, 0·25 mg. of xanthine and 0·25 ml. of 1/20,000 methylene blue were placed in a Thunberg tube which was then exhausted and incubated at 37°. No difference in time of decolorization was seen in comparison with blank tubes from which xanthine was omitted: there was thus no evidence that the virus possessed xanthine oxidase activity. The methylene blue was nevertheless decolorized over a period which ranged from 25 to 71 minutes, and the possibility still remained that this was an enzyme action involving some other substrate. To test this possibility an allantoic fluid with a haemagglutinin titre of 8,000 was divided into two parts: the decolorization time of the first part was determined without further treatment; the second part was adsorbed with red cells which were then removed by centrifugation. The haemagglutinin titre was now reduced to 40, showing that 99·5% of the virus had been removed; the decolorization time was now determined. A value of 110 minutes was found for the fluid both before and after adsorption of virus; whether the decolorization was enzymatic or not the virus particles were obviously not concerned in the reaction.

Virus was also tested for deoxyribonuclease activity. Allantoic fluid was placed in a viscometer with a solution of highly polymerized sodium deoxyribonucleate and veronal buffer of pH 7, and the viscosity of the mixture was observed over a period of 30 minutes. Four samples of allantoic fluid with haemagglutinin titres of 2,000 to 8,000 were tested in this way and no evidence of deoxyribonuclease activity was found. In another experiment which is illustrated in Fig. 1 the virus contained in

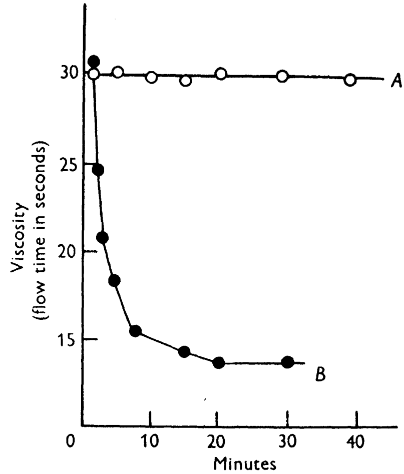

Fig. 1. Lack of deoxyribonuclease activity of influenza virus. A; PR8 virus from 140 ml. allantoic fluid concentrated by adsorption on red cells and elution into 2·5 ml. saline. The viscometer also contained 1 ml. sodium deoxyribonucleate (0·5% w/v), 1 ml. pH 8 veronal buffer, 1 ml. gelatine-magnesium diluent. The substrate is not depolymerized. B; the effect of 0·05 mgm. purified deoxyribonuclease. The substrate is rapidly depolymerized and the action is complete within 20 minutes.

140 ml. of allantoic fluid was adsorbed on to red cells and then eluted into 2·5 ml. of saline. No enzyme activity was found even with this concentrated preparation. The depolymerization produced by 50 μg.of crystalline deoxyribonuclease is illustrated in comparison.

Succinic dehydrogenase activity was tested by the methylene blue decolorization method; 1 ml. allantoic fluid, 0·5 ml. 1/20,000 methylene blue and 0·5 ml. pH 7 phosphate buffer were placed in a Thunberg tube with 0·5 ml. 0·05 M succinate in the side arm, and a control was set up with water in place of substrate solution. The tubes were evacuated, mixed and incubated at 37°, and the time of decolorization was noted. Some typical results are shown in Table 1. The amount of virus in the fluids was determined by the photoelectric method (Hirst & Pickels, 1942). The first two fluids showed no enzyme activity; the third showed a definite activity but this still persisted when most of the virus had been adsorbed. The results thus indicate that PR8 virus has no succinic dehydrogenase activity.

Table 1. *Lack of succinic dehydrogenase activity in influenza virus. The first two fluids did not decolorize methylene blue in the presence of succinate, and thus did not contain the enzyme. The third fluid showed dehydrogenase activity towards succinate, but this still persisted after the virus had been removed by adsorption.*

Fluid no.	Amount of virus in CCA units	Decolorization Time (minutes)		Enzyme Activity
		Test	Blank	
1	200	∞	∞	Absent
2	200	∞	∞	Absent
3	320	60	∞	Present
3 (adsorbed)	20	50	∞	Present

In further experiments allantoic fluid containing the virus in high titre had no adenosinetriphosphatase activity, no phosphatase activity against glycerophosphate or adenosine-5-phosphate and no nucleosidase activity against inosine.

As with vaccinia, the results obtained with influenza virus, although of limited extent, afford no reason for supposing that the virus possesses enzyme activity.

At this point some attention must be devoted to the mucinase activity of influenza virus, which apparently affords an exception to the general principle, which is in the process of development, that viruses are devoid of intrinsic enzyme activity. An enzyme action has been postulated to explain the facts of red cell agglutination and subsequent elution; it is suggested that influenza virus becomes attached to a specific substrate present on the surface of red cells and the cells consequently agglutinate by the formation of virus bridges. The substrate is then destroyed, the virus separates from the cells and so elutes, and the cells no longer agglutinate. The cells moreover cannot be agglutinated again by repeated exposure to virus of the same type, as the receptors are presumed to have been destroyed. A parallel is drawn between this sequence of events and the action of receptor-destroying enzyme upon red cells. This is an enzyme of mucinase type present in filtrates of cultures of *Vibrio cholerae*. When red cells are treated with this enzyme they can no longer be agglutinated by influenza virus, since the substrate to which the virus becomes attached is destroyed by the receptor-destroying enzyme. Treatment with the enzyme also renders the cells of the chorioallantoic membrane and the epithelium of the mouse lung unable to adsorb virus, and the enzyme also destroys the mucoid inhibitors of influenza virus haemagglutination which are present in serum, egg-white and elsewhere. The action of receptor-destroying enzyme is clearly mucolytic, and is considered by the proponents of the enzyme theory of influenza virus action to be directly comparable with the action of the virus.

It cannot be said, however, that the enzymic action of influenza virus action has been proved beyond doubt. Many of the experiments upon which it is based have been conducted with a serological approach and the matter has yet to be subjected to an examination with full enzymological rigour. One striking defect of the theory is that no alternative explanations of the phenomena have been seriously considered. For instance, the attachment of virus particles to the red cell membrane might reasonably be supposed to affect its permeability and thus upset the Donnan equilibrium, which controls the relative proportions of ions on either side. This disturbance could cause a compensatory migration of ions which would affect the total charge of the red cell and thus weaken the attractive forces between cell and virus. Elution of virus could then occur and the electrophoretic mobility of the cell would be reduced in conformity with the experimental findings. The red cell would moreover remain inagglutinable until such time as the original ionic balance had been restored. This possibility does not seem to have been tested experimentally, and it is indeed tacitly assumed that the red cell remains in a permanent state of inagglutinability. The concentration of ions in the environment is in fact known to be of prime importance in the haemagglutination produced by other viruses. The phenomena of agglutination and elution can be exactly reproduced with MM virus by appropriate alterations in the chloride concentration of the medium (Hallauer, 1951), and there is here no suggestion that an enzyme action is taking place. Much of the behaviour of influenza virus could also be explained by assuming that the virus consists of many layers of dissociable subunits (possibly soluble antigen) deposited upon a central core. These sub-units would have an affinity for mucoids which would cause the virus to adhere to red cells and thus agglutinate them. Thermal agitation of the relatively massive red cell and virus would disrupt the union, causing elution of the virus and disappearance of agglutination. The virus would still be coated with sufficient of the dissociable sub-units to be able to carry out further agglutination, whereas the mucoid receptors of the red cell would be masked with a layer of virus material which would render them inagglutinable. The interaction between virus and mucoid inhibitors could be of a similar nature, and it is reasonable to suppose that heating the virus could alter it in such a way as to render the superficial sub-units non-dissociable, so that the virus, while still capable of causing haemagglutination, would not be able to elute, and would also lose 'enzymic' activity against mucoids and therefore be in he so-called indicator state. It is not intended to imply that either of the above two theories represents the true state of affairs; they are mentioned with the sole purpose of showing that there are, besides the enzyme theory, at least two and possibly numerous other ways of explaining many of the observed facts which have not yet been seriously considered. The part played by the enzyme activity of influenza virus in natural infection is in any case doubtful, since it can be shown that when issue cells are treated with periodate they are no longer susceptible to enzyme action although they can still be infected in the ordinary way

Table 2. (*Modified from Bauer, 1949*). *The occurrence of enzymes and other metabolic factors in the elementary bodies of viruses*

Virus	Absent	Present	Comments
Vaccinia	Zymohexase Enolase Phosphoglucomutase Adenosine nucleosidase Peptidase Triosephosphate dehydrogenase		
		Phosphomonesterase Phosphodiesterase Ribonuclease Deoxyribonuclease	Probably absorbed on to virus from tissue of origin
	Cytochrome oxidase Cytochrome *c*		
		Riboflavine Copper Biotin	Probably an integral part of the virus body
Influenza		Mucinase	Legitimate doubt as to whether this is an enzyme action
	Deoxyribonuclease Phosphatase Xanthine oxidase Adenosine triphosphatase Succinic dehydrogenase		
Other Viruses			Not yet investigated

(Fazekas de St. Groth, 1948). It would be out of place in the present review to criticize the mucinase theory further, or to propose at this stage any theory to take its place; it is sufficient to note that there are solid reasons for doubting that the haemagglutinating and related activities of influenza virus are enzymatic in nature, and that the existence of these activities does not therefore constitute a serious objection to the thesis that viruses are devoid of enzyme activity.

The present state of knowledge concerning the biochemical properties of viruses is summarized in Table 2. Apart from the questionable instance of mucinase the investigations made so far suggest that viruses have no independent metabolic activities. This conclusion is based upon an incomplete examination of two viruses only: in the absence of proof to the contrary it is commonly extended to apply to all viruses and this may well be true, but it should be borne in mind that the doubt exists and will not be resolved until such time as adequate purification methods have been worked out for all viruses and utilized in exhaustive studies of metabolism. Nevertheless, with the above reservation, it will be considered in the following sections that absence of enzyme action is a fundamental property of viruses, and is a factor of prime importance in the consideration of the method by which they multiply.

THE METABOLISM OF TISSUES INFECTED WITH VIRUSES

The negative results of the investigation of the metabolism of viruses *in vitro* caused attention to be shifted to a study of the metabolism of viruses in their intracellular environment. This aspect of the subject has been limited by the extent of biochemical knowledge and has advanced hand in hand with it, and may be expected to yield results of prime importance in future work.

As with studies on isolated viruses, work with infected tissues was at first limited to the observation of respiratory activity. The Q_{O_2} of the brain and spinal cord of rhesus monkeys infected with an unnamed strain of poliomyelitis virus was studied by Brodie & Wortis (1934). Minced tissue was placed in Warburg vessels in Ringer alone or with glucose; Q_{O_2} values of 0·65 and 0·68 were found for infected tissue, compared with 0·82 for normal tissue. These values are very low and it is doubtful if the oxygen uptake observed was the maximum attainable. The subject was not pursued further until 1942, when Racker and Kabat determined the oxygen uptake and anaerobic glycolysis of a suspension of mouse brain infected with Lansing virus. The oxygen consumption of infected tissue showed no significant difference from normal, but the anaerobic glycolysis, expressed as output of carbon dioxide per 100 mg. tissue per hour, was significantly reduced below normal values. Victor & Huang (1944) investigated the effect of infection with western equine encephalomyelitis virus upon the anaerobic glycolysis of tissue cultures of minced chick embryo; their object was to develop a shortened titration procedure, but their results are directly concerned with the metabolism of the infected tissue. The values obtained for the rate of anaerobic glycolysis of uninfected control tissues showed great variation, but the figures for infected tissue were always lower than normal values. The results suggest that anaerobic glycolysis is inhibited during the course of the virus infection, although it is possible that co-factors might have become depleted during the course of incubation of the cultures, in which case the glycolysis measured might not represent the maximum detectable. Nickle & Kabat (1944) confirmed the previous observations of Racker & Kabat in an investigation of the respiration of mouse brain infected with Lansing and western equine encephalomyelitis viruses. A wide variation was found in the rates of oxygen uptake and anaerobic glycolysis, but the anaerobic glycolysis of the infected brains was apparently significantly less than normal in the presence of a high concentration of glucose, but was reduced only in the case of western equine encephalomyelitis virus when a low concentration of glucose was present. The authors concluded that a difference in metabolism had been observed. This work and the previous work of Racker and Kabat was repeated by Wood, Rusoff & Reiner (1945), who could find no significant difference between the values for normal and infected brain, and later (Utter, Reiner & Wood, 1945) pointed out that the amount of anaerobic glycolysis measured was far below the maximum value on account of random deficiencies in co-factors; this objection applies also to all the work done up to this time. Maximal production of carbon dioxide was only obtained when the brain suspension was fortified with hexose diphosphate, adenosine-triphosphate, diphosphopyridine nucleotide, nicotinamide and magnesium ions. Their objections may have been too sweeping, however, since in the light of present knowledge it is possible that infection with a virus might influence an enzyme action by depleting the store of co-factors, and the study of simple unfortified extracts could be made to afford information as to the actual level of metabolism during the course of virus infection as opposed to the potential maximum.

Pearson & Winzler (1949) investigated the metabolism of fragments of mouse brain infected with GDVII virus and incubated in Warburg vessels for up to 70 hours. The oxygen consumption fell off very rapidly and had reached very low values at the end of the experimental period;

there was no significant difference from the values observed with uninfected tissue, nor was there any difference in the rate of glycolysis. The authors stress the fact that the observations were made at a time when the rate of virus growth was maximal and therefore show that respiration is unaffected by the growth of virus in the tissue. Whereas the rate of multiplication of the virus is admittedly high, the total amount of virus present in the first part of the experimental period is very low, and since the virus multiplies in a logarithmic manner may actually be considered as negligible from the point of view of any effect that it could have upon metabolism. Observable changes in metabolism can only be expected when the amount of virus present, in other words its titre, has reached a maximum, and by the time this had occurred in the experiments of Pearson & Winzler the respiration of the tissue had already fallen to very low levels on account of the inevitable deterioration of the preparation.

Attempts have been made to observe metabolic changes in fertile eggs infected with viruses. Parodi *et al.* (1948) incubated eggs at 37° in vacuum desiccators filled with air, and followed the respiratory exchanges by gas analysis. Eggs which had been inoculated with a strain of influenza A virus isolated in the Argentine showed an increase in oxygen uptake after 24 hours, but after 48 hours the average oxygen uptake and carbon dioxide output of infected eggs were lower than those of normal fertile eggs of the same age and duration of incubation. When the same results were expressed per gram of embryo, however, no significant difference from normal values was found. Pinkerton *et al.* (1950), using a similar technique, determined the virus content and oxygen consumption of eggs inoculated with PR8 virus at intervals up to 96 hours after infection. The rate of oxygen consumption rose during the course of incubation, but fell off after 24 hours at a time when the titre of virus was attaining a maximum; further examples of parallelism between respiration and titre were noted in experiments carried out at different temperatures of incubation. The authors suggest that the transient depression of respiration is due to a toxic effect of newly liberated virus. In further work (Greiff, Blumenthal & Pinkerton, 1950) the oxygen consumption of eggs was determined 90 minutes after the intra-allantoic injection of allantoic fluid containing PR8 virus. The oxygen uptake was increased above normal values when up to 2 ml. of fluid were injected, but in eggs injected with 3 ml. the uptake of oxygen was depressed. These effects were abolished when the allantoic fluid was previously heated at 56° for 30 minutes, and were considered to be due to the action of a toxin.

McLimans, Siem & Scholljegerdes (1950) and McLimans *et al.* (1950) carried out similar observations with Newcastle disease virus, using a technique in which single eggs were incubated in a specially designed Warburg chamber. The oxygen uptake per egg showed no departure from normal until two days after infection with the virus; it then fell rapidly to zero during the terminal stages of the infection when death of the embryo occurred. Similar results were obtained in experiments with western equine encephalomyelitis virus (Siem, Smith & McLimans 1950); eggs inoculated with the virus showed a normal rate of oxygen consumption for 17 hours, when the rate rapidly fell to zero as death of the embryo occurred.

The work on entire eggs carried out so far has yielded no information of any value. Multiplication of the viruses which have been used is confined to the embryo or the chorioallantoic membrane; any changes which occur in the respiration of these tissues will probably be obscured by the respiration of the remainder of the egg, and the method is thus not likely to yield results of any significance. Much more could be achieved by a study of the metabolism of isolated pieces of membrane, which could be infected *in vitro*, or by previous inoculation of the egg at a predetermined time. In personal observations, so far unpublished, it was found that influenza virus increased the oxygen uptake of allantoic membrane. Pieces about 10 mm. square were cut from the same membrane and placed in Warburg flasks containing serum Tyrode and infected or normal allantoic fluid. The flasks were incubated at 37° and the oxygen uptake was observed and expressed as cubic mm. consumed per mg. dry weight of tissue per hour (Q_{O_2}). Values of 10·0 and 6·25 were found for membrane alone and 10·5 and 8·3 with the addition of 1 ml. of normal allantoic fluid, whereas values of 54·1 and 28·3 were found for membrane in the presence of 1 ml. of allantoic fluid containing PR8 virus to a titre of 8000 by the Salk test. The results of a similar experiment are recorded in Table 3, which shows the effects of a fluid before and after adsorption of virus, and also of the adsorbed virus after elution into Tyrode solution. In these experiments an interval of 20 minutes elapsed before adding virus to the membrane and taking readings, and the Q_{O_2} values are based on observations over a period of two hours. The results thus show that a marked rise in Q_{O_2} occurs within two hours of adding infected allantoic fluid to the membrane. In the experiments of Table 3 the rise was less marked when part of the virus had been removed by adsorption, but on the other hand the eluted virus produced a still lesser effect. The stimulation of the oxygen consumption of the membrane observed here is in conformity with the findings of Greiff *et al.* (1950). Their suggestion that

Table 3. *The effect of PR8 virus upon the Q_{O_2} of chorioallantoic membrane The Q_{O_2} is increased by the addition of chorioallantoic fluid containin the virus; the increase is less marked when the virus is partially remove by adsorption.*

Addition	Titre of virus added	Q_{O_2}	Comment
Unadsorbed PR8 fluid	4000	43·6, 41·5	Q_{O_2} increased
Partially adsorbed fluid	2000	30·5, 27·5	Increase less marked
Eluted virus	8000	13·1, 22·2	Increased ×3
None	—	4·4, 7·0	Normal level of membrane respiration

the effect is due to a toxic action of the virus may of course be true, bu in any case it depends upon the interpretation of toxicity. When viru is added to the membrane it is rapidly adsorbed, and any changes i metabolism which are observed and which may be ascribed to toxicit are more likely to represent a specific effect of the virus upon cell metabolism and a necessary stage in the cycle of virus development. Th isolated membrane infected *in vitro* is a very convenient model for studying virus metabolism. Enough pieces can be cut from the same membrane to afford an adequate number of replicates, and various enzym reactions can be studied by adding the appropriate substrates. It is o course essential to add an excess of virus so as to infect all the availabl cells and thus achieve the maximum observable effect upon metabolism In the case of influenza virus the experimental results can be very easil controlled by comparison with the effects of allantoic fluid from whic the virus has been specifically adsorbed. Once a satisfactory techniqu has been established an extensive application of the method should yiel results of value, as all the metabolic events occurring in a single cycle o virus reproduction could be recorded and correlated with the appearanc of soluble antigen and infectious virus in the membrane.

In conclusion the work of Kun & Smith (1950) may be mentioned Determinations were made of the Q_{O_2} of membranes which had been infected with myxoma virus by inoculating the egg 48 hours previousl No difference from normal values was found. Significant difference were found, however, in certain specific enzyme activities, and these results will be considered later in the appropriate context.

The results of respiration studies have so far yielded little informatio of value, but much more progress has been made in studies of individua enzyme reactions in tissues infected with viruses. Racker & Kabat (1942) in their work on the respiration of mouse brain infected with Lansing virus also investigated dehydrogenase and phosphatase activity. In experiments with the Thunberg technique suspensions of infected brain decolorized methylene blue more rapidly than suspensions of normal brain, but no difference was observed when various substrates such as glucose, lactate and succinate were added. There was no difference in phosphatase activity, but adenosinetriphosphatase activity was increased in infected brains. Experimental details are not given, and these results must be regarded with some reserve; in order to obtain a true measure of phosphatase activity in extracts the determination should be made in the presence and absence of magnesium ions, and determinations of adenosinetriphosphatase activity should similarly be made in the presence and absence of calcium ions, and the enzyme activity derived in either case from the difference between the two sets of readings. Unless these precautions are observed it is difficult to know precisely what activity is being measured.

Racker & Krimsky (1946) studied the phosphorylation of glucose in mouse brain infected with Lansing & Theiler viruses. Brain homogenates were incubated for one hour with glucose, adenosinetriphosphate and diphosphopyridine nucleotide, together with nicotinamide as an inhibitor of the enzyme system which destroys the latter substance, and the amount of glucose utilized at the end of the period of incubation was determined by titration. In both virus infections the utilization of glucose was below normal values; brain infected with Lansing virus showed a reduction of glycolysis ranging from 0 to 50%, with an average of 15%, and brains infected with Theiler's virus showed a reduction of 0 to 80%, with an average of 31%. Reduction in glycolysis was also seen when glucose-6-phosphate and fructose-6-phosphate were used as substrates instead of glucose, but not when hexose diphosphate was used. A similar inhibition of glycolysis was obtained when partially purified preparations of Lansing and Theiler FA viruses were added to a homogenate of normal mouse brain acting upon substrate in the presence of the same co-factors. Purified preparations of PR8 virus and tobacco mosaic virus also inhibited the glycolysis of normal mouse brain. In further work (Racker & Krimsky, 1947) the inhibition of normal mouse brain glycolysis by purified preparations of FA virus was investigated in greater detail. The same degree of inhibition was obtained with virus preparations which had been boiled or incinerated, and the inhibitory effect was found to reside in the iron contained in the preparation. The authors concluded that FA virus inhibited glycolysis by the mediation of ferrous ion, which is either part of the virus body or else is liberated during the course of infection. It is not possible to assess these results without much further work. The observations may be of fundamental importance or they may be quite irrelevant, according as to whether iron is an integral part of the virus bodies or a contaminant derived from the tissue of origin. With the example of the copper content of vaccinia virus in mind it is quite conceivable that iron could be an integral part of the virus. If this were the case infected brain should have a higher iron content than normal brain, a point which might be worth investigation.

Bauer (1947*a*) investigated the xanthine oxidase activity of mouse brain during the course of infection with the 17D strain of yellow fever virus. The activity of the enzyme increased steadily during the incubation period and reached a maximum on the seventh day after infection, when symptoms of encephalitis appeared. A similar increase in xanthine oxidase activity was also seen in brains infected with the neurotropic and Asibi strains of yellow fever, and with lymphocytic choriomeningitis. The increase in xanthine oxidase activity was not peculiar to mouse brain, but was also seen in chick embryos infected with the 17D strain of yellow fever virus (Bauer, 1948). The xanthine oxidase content of the normal embryo is very low, and it is possible that the considerable increase seen during the virus infection represents a formation of new enzyme. Further studies were made of the dehydrogenases of mouse brain (Bauer, 1947*b*). A marked increase in xanthine oxidase and pyruvic dehydrogenase activities occurred during infection with neurotropic yellow fever, lymphocytic choriomeningitis and lymphogranuloma inguinale. A lesser rise in dehydrogenase activity was also seen when succinate, lactate and malate were used as substrates. The results thus indicate that there is an increase in the activity of the cyclophorase system during virus infection. Studies were also made of the activity of some of the enzymes concerned in nucleic acid metabolism during the course of infection with the viruses of neurotropic yellow fever, lymphocytic choriomeningitis, neurovaccinia and dengue (Bauer, 1951*a*). The enzymes studied included deaminases, nucleosidases and nucleotidases; the activities of some enzymes showed no difference from the average values for normal mouse brain, but the activities of other enzymes were found to be increased or decreased. The results obtained are shown in Table 4. The pattern of alteration of enzyme activity is such as to suggest that certain metabolic pathways leading from purines and pyrimidines to nucleic acids are quantitatively insufficient and are specifically expanded during the virus infection. Thus in the case of yellow fever the activities of adenosine deaminase, xanthine oxidase and the nucleosidases for inosine and xanthine are all increased; there is thus increased metabolic activity in the pathways leading from xanthine to adenosine and xanthosine respectively. It is probable that a more thorough study of enzyme activities would give some indication of the reactions which are involved in the synthesis of virus material, but in the present context it is sufficient to note the fact that enzyme activity is affected during virus multiplication.

Kun & Smith (1950), in an extension of their work on the respiration of the allantoic membrane infected with myxoma virus which has already been reviewed, also observed that zymohexase activity was increased and lactic acid production was correspondingly increased above normal values; the average *Q* value for lactic acid production of infected membrane was 40·6, compared with an average value of 16·0 for uninfected membrane. The authors suggest that zymohexase is specifically activated by the virus, and their results are thus a further example of the increase of enzyme activities seen in virus infections of mouse brain which have already been described.

Little & Lawson (1951) investigated the cholinesterase activity of mouse brain infected with Theiler FA and Lansing viruses. Homogenates of infected brains, and the supernatant and sediment obtained from them by centrifugation, were incubated in Warburg flasks with acetylcholine at pH 7·4 with 95% nitrogen and 5% carbon dioxide in the gas phase, and the hydrolysis of acetylcholine was followed from the rate of liberation of carbon dioxide. The results indicated that the cholinesterase activity of homogenates and sediments of brain infected with FA virus was significantly reduced, whereas no change was seen in the case of brains infected with Lansing virus. It is concluded that the decrease in enzyme activity was not sufficient to affect conduction and therefore account for the symptoms of encephalitis. The results must be viewed with some suspicion, however, since it is by no means certain that the carbon dioxide evolved had been liberated by the breakdown of acetylcholine. Under the conditions of the experiment brain extracts will carry out a certain amount of glycolysis with the formation of lactic acid and consequent liberation of carbon dioxide; acetylcholine salts give an acid solution which would itself liberate carbon dioxide from bicarbonate buffer, and it is not stated whether the solutions were neutralized before use. In any case the amount of carbon dioxide evolved seems very large; the results indicate that 10 mg. of acetylcholine were hydrolysed per hour, whereas Bauer (1951*b*) found that a suspension of mouse brain which was five times more concentrated hydrolysed only

0·75 mg. of acetylcholine per hour at the more favourable pH of 8·5. In this work the hydrolysis of acetylcholine was followed by titrating the amount of acetic acid liberated; the pH was maintained at the optimal value of 8·5 by borate buffer and incubation was carried out at 37° for two hours. The non-enzymatic decomposition of acetylcholine which occurs in alkaline solutions was determined by titration of duplicates to which sufficient eserine had been added to inhibit cholinesterase activity, and the activity of the enzyme was obtained from the difference between the two titration figures. In brains infected with neurotropic yellow fever, neurovaccinia and MM viruses cholinesterase activity was significantly lower than normal values, whereas there was no difference from normal in infections with lymphocytic choriomeningitis and Lansing viruses. In brains infected with the neuro-WS strain of influenza virus cholinesterase activity was increased above normal values. The depression of cholinesterase activity bears some relation to the course of multiplication of the virus, since in infections with neurotropic yellow fever virus the enzyme activity falls gradually during the incubation period and reaches a minimum value at the time when symptoms of encephalitis appear (see Fig. 2). These observations open up a subsidiary line of investigation of some interest. The choice of mouse brain as a test object,

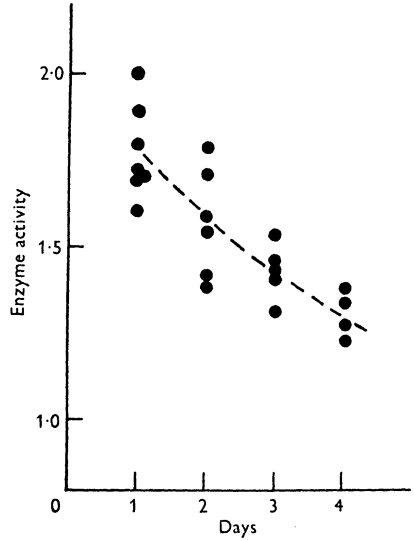

Fig. 2. Cholinesterase activity of mouse brain during infection with neurotropic yellow fever virus. Mice were inoculated with 1000 LD_{50} on day 0 and determinations of cholinesterase activity were made on the brains of mice sacrificed at intervals. Symptoms of encephalitis appeared on the 4th day. The line passes through the average values.

while permitting the investigation of a large number of viruses, also affords the possibility of examining a number of metabolic factors which, while they may be of fundamental or only of incidental importance for virus multiplication, are intimately connected with maintenance of nervous function, and any deficiency of which may cause the appearance of specific symptoms. For instance, encephalitis in the mouse tends to appear in one of three forms; a generalized flaccid paralysis, as with dengue, Lansing virus and the 17D strain of yellow fever virus; cerebral irritation, as with neurotropic yellow fever, MM and neuro-WS viruses; and excitation with convulsions, as with lymphocytic choriomeningitis and neurovaccinia. It is possible that some differences might be found

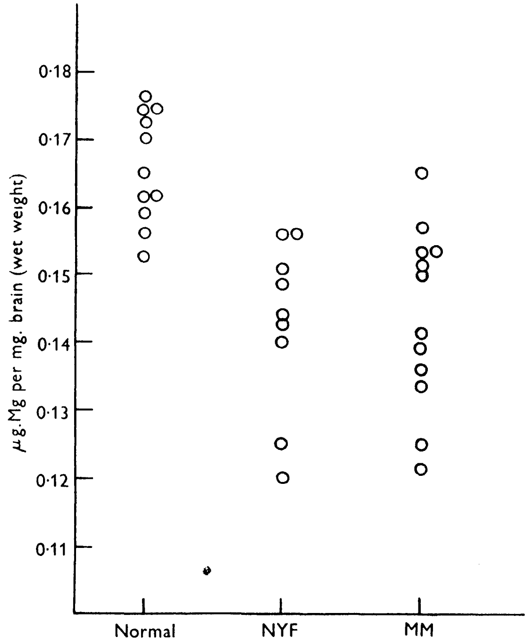

Fig. 3. The effect of virus encephalitis upon the magnesium content of mouse brain. Lower values are found in infections with neurotropic yellow fever and MM viruses.

associated with these different types in those factors, such as cholinesterase, choline acetylase, acetylcholine content, and content of magnesium, thiamine and glucose, which are responsible for the maintenance of a normal level of nervous activity. Bauer (1951*b*) found that the magnesium content of mouse brain was depressed below normal values in infections with neurotropic yellow fever and MM viruses (see Fig. 3), and this deficiency of magnesium might be the cause of the nervous hyperactivity which is characteristic of this type of encephalitis. Whether the shift of magnesium is essential for the metabolic processes involved in virus multiplication or whether it is an incidental consequence of cell damage awaits further elucidation. Information of this sort is, however, of practical importance and might even have some place in the symptomatic treatment of virus encephalitis.

The changes in enzyme activity and other factors of metabolic importance which have so far been shown to occur in tissues infected with viruses are summarized in Table 4. A further point which emerges from the study of the table is that the pattern of activation or inhibition is not the same for all viruses. There is, for instance, an increase in the activities of adenosinetriphosphatase and the nucleotidase for adenosine-5-phosphoric acid in infection with vaccinia virus which does not occur in infections with the other viruses so far studied, and other instances of the same phenomenon may be noted as well. Viruses thus appear to show a certain diversity in what may be termed their biochemical properties, which may be an expression of specific differences in the synthetic processes utilized in the production of viruses of different types.

NUTRITIONAL FACTORS IN VIRUS INFECTIONS

From the work described in the preceding section it appears that the occurrence of virus multiplication is associated with the appearance of certain changes in the metabolic activity of the host cell, and it follows from this that any factors which influence the activity of the host cell as a whole may also affect the growth of virus within it. Here must be considered factors which affect the nutrition of the cell, such as vitamin deficiencies and dietary restrictions, and factors which affect its overall activity, such as hormones and the age of the host tissues, and a further fact which needs to be taken into consideration is the relative or absolute insusceptibility of some tissues and animal species towards infection with a particular virus.

The effect of thiamine in controlling virus growth was investigated by Rasmussen *et al.* (1943). Mice were placed on diets containing adequate and insufficient amounts of thiamine respectively, and a comparison was made of the mortalities in the two groups after infection with Theiler FA virus. Little difference was found, but in a similar experiment with Lansing virus all of 35 mice receiving a thiamine-deficient diet escaped paralysis, compared with 10 of 35 in the control group. Similar experiments were also carried out on the effect of riboflavine deficiency on infection with Theiler virus, but no significant difference was observed. The effect of thiamine deficiency upon infection with Lansing virus was also investigated by Foster *et al.* (1944). They found that only 9% of 77 mice on the deficient diet were paralysed on the

Table 4. *The effect of virus infections upon the enzyme activities and other metabolic factors of the host tissues. Abbreviations: NYF, neurotropic yellow fever; LCM, lymphocytic choriomeningitis; NV, neurovaccinia; LGI, lymphogranuloma inguinale; D, dengue; I, influenza.*

Virus	Enzyme or Other Metabolic Factor					
	Dehydrogenases					
	Xanthine oxidase	Succinate	Lactate	Pyruvate	Malate	Glycerophosphate
NYF	+	+	+	+	+	—
LCM	+	+	+	+	+	—
NV	+					
LGI	+	+	+	+	+	—

	Deaminases			Nucleotidases	
	Guanosine	Adenosine	Guanase	Adenosine-5-phosphoric acid	Guanylic acid
NYF	0	+	0	—	—
LCM	0	+	0	0	0
NV	—	+	0	+	0
D	—	+	0	0	0

	Nucleosidases				
	Inosine	Uridine	Guanosine	Xanthosine	Adenosine
NYF	+	+	0	+	0
LCM	+	+	0	0	0
NV	0	+	0	0	0
D	+	+	+	+	+

	Adenosine-triphosphatase	Cholinesterase	Magnesium content	Virus	Oxygen uptake	Zymohexase	Lactic Acid Formation
NYF	0	—	—	Myxoma	0	+	+
LCM	0	0					
NV	+	—		I	+		
D	0	0					
MM		0	—				

+ Increase in activity of enzyme or concentration of factor.
— Decrease in activity of enzyme or concentration of factor.
0 No change in activity of enzyme or concentration of factor.

twenty-first day after infection, whereas 75% of 79 mice receiving an adequate diet had become paralysed by this time. There was no difference in the eventual mortalities in the two groups, however. In further experiments with groups of from 22 to 166 mice a reduced mortality was noted at 21 and 46 days in the mice receiving the deficient diet, but by 59 days the mortalities in the test and control groups showed no significant difference. The authors concluded that a deficiency of thiamine delayed the action of the virus but was unable to prevent its multiplication. Animals deficient in thiamine suffer from anorexia, and it was found that a restriction of 40% in the total amount of food taken had the same effect upon the course of the infection. It is thus not clear from this work whether the effect of thiamine is specific or not.

The effect of temperature upon virus infection was investigated by Holtman (1946*a*). Mice were acclimatized to temperatures of 13°, 22° and 32°, and were then inoculated with an unnamed strain of poliomyelitis virus. The incubation period depended in a very marked degree upon the temperature of acclimatization, and ranged from 5 days in the animals kept at 32° to 13 days in those kept at 13°. In further work (Holtman, 1946*b*) the possibility was considered that this was mediated through the effect of environmental temperature upon the activity of the thyroid. Mice were infected with poliomyelitis virus and treated with thiouracil, thyroid extract and iodinated casein. The incubation period was reduced in mice treated with thiouracil, but was increased by treatment with thyroid extract and iodinated casein. It thus appears that poliomyelitis virus may require a certain restricted range of metabolic rates for optimal multiplication. In the case of MM virus, however, Gollan (1948) found that treatment with thyroxin had no effect upon the course of the infection.

The rate of growth of the experimental animal might also be expected to influence the course of virus development. Kalter (1949*b*) placed young mice on diets which allowed rapid, moderate and no growth to occur, and then carried out a titration of a preparation of PR8 virus in all three groups. No difference was found in the titres observed. The effect of the age of the mice was also investigated (Kalter, 1949*a*). A preparation of PR8 virus was titrated in mice of ages ranging from 3 to 25 weeks. A titre of 10^6 was observed in the youngest mice, and the titre fell off evenly with increasing age to reach a value of 10^2 in mice 25 weeks old. Similar results were obtained with the Lee and Swine-15 strains. The final amount of virus in the lungs was nevertheless the same in mice aged 3 and 30 weeks, as determined by egg inoculation, and it was concluded that the older mice must be less susceptible, since they survived a concentration of virus which was lethal for younger animals. It is quite possible, however, that the lungs of the younger mice contained non-infective virus of a further cycle of multiplication, and that this additional virus brought about the death of the animals before developing to maturity and thus becoming detectable by infectivity titration. If this is the case the results of Kalter suggest that the virus multiplies to a lesser extent in older mice. Kalter, Stuart & Tepperman (1950) attempted to influence the rate of virus multiplication by controlling protein anabolism with hormones, thus simulating the effect of age upon the host tissues. Mice were treated with pituitary growth hormone or testosterone and were then inoculated intranasally with PR8 virus; the rate of growth of virus was determined by inoculation of eggs with lungs of mice sacrificed at intervals. A greater amount of virus, amounting to 1 to 2 log. units, was found in treated animals as compared with controls.

The fact that age of the host influences the growth of viruses is also common knowledge among virus workers, quite apart from researches specifically concerned with this point. The Coxsackie viruses, for example, will produce a fatal infection in infant mice, whereas older mice appear to be insusceptible. A preparation of dengue virus will show a titre of 10^5 in infant mice, but only 10^3 or 10^4 in mice of 4 weeks of age. The mice which are usually used in virus work are only a few weeks old, and it is well known that elderly mice show a reduced susceptibility to infection with viruses to which they can have no natural immunity.

Cortisone also appears to have a profound effect upon the course of virus infections. Schwartzman (1950) found that treatment with cortisone would shorten the incubation period of the MEF1 strain of poliomyelitis virus in mice, and hamsters, which are otherwise almost insusceptible to the virus, succumbed to a prompt and violent infection after treatment with cortisone. Kalter *et al.* (1951), however, found that treatment with cortisone reduced the amount of PR8 virus in the lungs of mice, and ascribed this effect to a decrease in protein anabolism brought about by the hormone. Cortisone is evidently an important factor in controlling virus growth, but it remains to be seen whether its effect is specific, or whether it merely depends upon an overall control of cell metabolism, or even on a facilitation of the spread of the virus from one cell to another.

As a final example of the way in which virus growth is dependent upon the host cell mention may be made of tropisms and tissue and species specificity. The tropisms of viruses were among the first of their properties to attract notice; less attention is perhaps paid to tropisms nowadays when practically all viruses can be adapted to growth in mouse brain, but it is nevertheless obvious that viruses tend to favour specific tissues as sites of multiplication, presumably because they require, at least in their unadapted forms, a metabolic climate which is not generally available. Another striking feature is the relative or complete inability of certain tissues to support virus multiplication, which may in fact be considered as a negative tropism. This insusceptibility may apply only to certain viruses, for example dengue and yellow fever viruses will not multiply in mouse liver, although Rift Valley fever and lymphocytic choriomeningitis viruses will do so, or it may apply to certain tissues, which are refractory to all viruses. For instance, virus infections of the skin, brain, lungs and liver are common, and the cells of these tissues presumably afford a metabolic environment which is generally suitable for viruses, whereas virus infections of the thyroid, adrenals, pituitary and possibly the kidneys do not seem to occur. It can only be concluded that the cells of these tissues by virtue of their specialized nature possess a metabolic environment in which the virus cannot survive.

CONCLUSION

In the preceding sections the information available up to the present time upon the metabolic events which are concerned in the growth of viruses has been set forth, and the bearing of this information upon theories of virus multiplication will now be examined. It may be assumed, so far as is known at present, that viruses do not possess any independent enzyme activities of their own. It necessarily follows from this that the substances which are required for the formation of new virus material must be supplied by the activity of the host metabolism. It is not likely that these substances are already present in the cell in adequate amounts, since the formation of nucleoprotein, an important constituent of the virus body, is not a normal activity of the resting cytoplasm, and it is thus not surprising to find that disturbances of metabolism can be detected in cells infected with viruses. The general pattern of the disturbance will only become apparent when many more enzyme activities have been investigated, but it has already been established that the activities of enzymes may be stimulated or inhibited, that the disturbance affects certain enzymes only and leaves others unaltered, and that the actual identity of the enzymes affected varies from one infection to another and is possibly a specific property of the viruses concerned. The disturbance of metabolism is thus apparently selective, and its occurrence implies that virus multiplication is brought about by the assumption of an overriding control of cytoplasmic activity by a foreign agency. Before discussing the nature of this agency, and considering whether it is in fact so foreign as it seems, we must first consider the methods by which a control of enzyme activities might be achieved. An increase in activity might be due to synthesis of new enzyme, and an instance has already been quoted (Bauer, 1948) in which this is a possible explanation. The level of enzyme activity in the intact cell depends not only upon the concentration of substrates, but also upon the concentrations of specific co-enzymes and ions, and control of activity might be achieved by a mechanism which could donate or withdraw the necessary activators or inhibitors. The concept thus emerges of the virus as an enzyme inactivator and inhibitor, but at what may be termed a 'higher than co-factor' level. The demonstrable presence of copper, biotin and flavine in vaccinia virus lends some support to this concept, and the loss of magnesium in infections with neurotropic yellow fever and MM viruses shows that ionic shifts are a feature of virus infections, although there is as yet no evidence as to whether they are incidental occurrences or not. The fact that some enzymes are inhibited at the same time as others are activated suggests that there is a redistribution of co-factors from a pool which is not inexhaustible, so that the gain of one enzyme must be offset by the loss to another.

Any explanation of the means by which enzymes are activated and inhibited by the virus must take into consideration the fact that the enzymes are scattered throughout the whole of the cytoplasm and are mostly located in discrete bodies, the mitochondria and microsomes. Methods for the isolation of these cytoplasmic constituents have been developed, and it would be of interest to separate cells infected with a virus into nuclear, mitochondrial, microsome and residual fractions in order to see precisely at what site the enzyme changes are occurring. The direction of enzyme activity seen in a virus infection must thus be widely distributed throughout the cytoplasm, and could be exerted directly upon the enzyme-containing structures themselves, or indirectly through an effect upon the nucleus. If the views of Caspersson (1950) are correct it is possible that both mechanisms may be involved. He considers that the nucleolus forms histones which pass to the inner surface of the nuclear membrane, whereupon ribose nucleic acid and cytoplasmic protein are caused to be synthesized upon the outer surface. If the virus makes use of this normal cellular apparatus for protein and ribonucleic acid synthesis it must act via the nucleus. The nucleus is undoubtedly involved in virus infections, since intranuclear inclusions may occur. It has been pointed out (Bauer, 1949) that a consideration of the cycle yield of influenza virus suggests that the nucleus is affected. In one cycle 40 to 60 infective particles are liberated. From the relative sizes of the virus and the cells of the chorio-allantoic membrane it can be estimated that the virus which is formed represents $1{\cdot}5 \times 10^{-5}$ of the total volume of the cell. Even allowing for an error of 10 or 100-fold in the estimate of the number of particles formed, it is evident that the virus constitutes only a small fraction of the total volume of the cell, yet it is sufficient to cause its death. The inference is that virus multiplication involves a vital structure, for instance the nucleus. The nucleus might of course be damaged by a toxic action of the virus or of associated products of its multiplication, but these would need to possess an improbably high toxicity in view of the small mass of virus material actually present.

Whether control of metabolism is exerted through the nucleus or directly upon the cytoplasm the spatial separation of the synthesizing units which are involved makes necessary the supposition that the virus dissociates into a number of sub-units after it enters the cell, each of which could influence a single synthesizing centre and direct the formation of new virus material. This concept, based upon biochemical and cytological considerations, agrees well with the soluble phase theory suggested by Hoyle (1948) which has been developed from immunological evidence. He finds that influenza virus loses its identity as an infective unit after entering the cell, and cannot be detected as such until the end of the cycle of infection. During this time, however, soluble antigen appears in the cell and increases in amount, and probably represents an incomplete form of virus. It may be supposed that the soluble phase represents the organization of new virus material which follows the widespread penetration of sub-units derived from the original particles at the time when it loses its infectivity. The biochemical evidence upon which the present theory of virus multiplication rests has of course been derived from a study of other viruses than influenza, and Bauer (1949) was therefore led to suggest that the soluble phase might be a feature of viruses in general, putting forward in support the fact that a latent period in which the virus is either non-infective or else invisible has been observed in the case of a number of viruses, and that the production of soluble antigens appears to be a common and perhaps a general feature of virus infections.

It may be as well to summarize the conclusions arrived at so far by a concise statement of the theory of virus multiplication which has been derived from a study of the biochemical evidence. It is postulated that the virus particle, after entering the cell, breaks up into a large number of sub-units which diffuse through the cell and influence the synthesizing centres, either the mitochondria and microsomes, or the nucleus, or perhaps both. New virus material is laid down around the centres under the controlling influence of the sub-unit, exerted possibly by co-factor redistribution. The new virus material, together with the organizing sub-units, represents the soluble phase of Hoyle, and represents also the inclusions which can be seen in the cytoplasm in the case of certain viruses. The formation of particles out of the new virus material must now be considered. It is by no means certain that all viruses exist in a truly particulate phase. This may seem an astounding statement to make, in view of the fact that the particulate phase of the virus is the one with which we are most familiar, but considerable doubt has been cast upon its reality by recent work of Hoyle (1950). He has observed the outgrowth of filaments from the surface of cells infected with influenza virus; the filaments tend to become segmented into spheres, and both filaments and spheres have the dimensions regarded as characteristic of influenza virus particles and the filaments often seen in association with them. The same phenomenon was also observed with normal uninfected cells when they were placed in an unfavourable environment. It therefore seems probable that the particles of influenza virus are merely droplets of diseased cytoplasm which assume that shape from purely incidental reasons. A similar appearance has been observed in electron micrographs by Wyckoff (1951), who was also unable to find any particles resembling virus in an intracellular position. He also observed that discrete virus particles were formed only by well-adapted laboratory strains, and that cells infected with recently isolated strains of influenza virus underwent rapid necrosis and particles resembling virus bodies could be found only with difficulty. The larger viruses, such as those of the psittacosis and vaccinia groups, seem to form elementary bodies in intracellular colonies, but it is not impossible that the smallest viruses do not form discrete particles, for many of them have not been seen in electron micrographs, and although this may be due to difficulties of purification, and in the attainment of sufficiently high concentrations, together with the limit of resolution of the electron microscope, it is also possible that these viruses exist in the soluble phase only. Influenza virus may represent an intermediate condition, in which the virus exists as particles of soluble phase which are formed fortuitously from extracellular processes; it is possible that particle formation is not an essential stage in the life cycle of this virus, since recently isolated strains of influenza virus apparently cause necrosis of cells and liberation of the abnormal cytoplasm without the formation of filaments or particles, and yet these viruses still retain their infectivity. It is probable that further work will disclose other examples of what may be called spurious elementary body formation. In the case of influenza the formation of particles needs no further explanation than that given by Hoyle, and in the case of the viruses of the psittacosis and vaccinia type it has been suggested (Bauer, 1949) that each virus particle results from the condensation of virus material around an individual synthesizing centre.

The theory of virus multiplication outlined above bears no relation to exponential replication of bacterial type, and if this theory is accepted it carries with it the implication that viruses do not represent degenerated or highly specialized forms of bacteria, but are in fact totally unrelated to them. It may be useful at this point to tabulate some of the more striking differences between viruses and bacteria which have become evident in recent years (Table 5). The suggestion that viruses utilize the host metabolism is supported by the fact that virus growth can be controlled by factors such as host age, hormones and vitamin deficiencies, which affect protein synthesis in the host cells. These factors apparently take prior effect and set the metabolism at a certain level of activity which cannot be altered by the action of the virus. Bacteria, of

Table 5. *Comparison of viruses with bacteria and cell constituents in a broad sense on the basis of certain of their properties*

Property	Viruses	Bacteria	Cell constituents
Subject to overriding control of host protein anabolism	yes	no	yes
Influenced by host age and nutrition	yes	no	yes
Marked restriction of tissue and host range	common	not common	specific to the tissue and host
Control activity of host cell enzymes	yes	no	yes
Produce interference	yes	yes, but not of same type	inapplicable
Non-particulate phase	yes	no	yes, i.e. organizers, transmitters of gene influence
Size range	smaller than bacteria	larger than cell constituents	same range as viruses

course, are in no way affected by factors of this kind, and multiply without regard to the condition of the host metabolism. Instances are certainly known where the tissue and host range of a bacterium are limited, but this limitation is unusual among bacteria and is not nearly such a prominent feature as with viruses. Bacteria will interfere with the growth of other bacteria, but only by the production of antibiotic substances or by exhausting nutrients, and not at all in the manner characteristic of the interference phenomenon of viruses. Other differences between viruses and bacteria listed in Table 5 need no comment, and the significance of the fourth column will be discussed below.

The theory of virus multiplication by means of a soluble phase which directs cell metabolism can explain several important manifestations which are peculiar to virus infections. Subjection to the overall control of the host protein anabolic rate has already been described: intracellular virus directs the metabolic mechanism in the state in which it finds it, and cannot alter its level of operation which is preset by external influences. If protein anabolism is depressed, the growth cycle of the virus, and therefore the incubation period of the disease, must necessarily be prolonged. The interference by one virus of the growth of another, often completely unrelated, virus, can be explained in those instances where it occurs intracellularly by assuming that synthesizing centres of the host which are already under the control of one virus are rendered insusceptible to subsequent control by another. Recombination can be explained by assuming that the soluble phases of the two viruses become mixed inside the cell and show a greater or lesser degree of synergism in their action upon the synthesizing centres. The contrast between the crystalline and soluble nature of the plant viruses and the particulate and insoluble nature of the animal viruses has long been an enigma, but the distinction disappears with the postulation of a soluble phase as a general property of animal viruses, and with the recognition of the perfunctory nature of the particulate phase of influenza virus. The possibility that all neoplasms are caused by viruses might well be reconsidered in the light of present views on the nature of viruses and virus multiplication; the fact that a virus cannot be detected in a neoplasm by infectivity tests does not mean that it is not there, since it might exist permanently in the soluble phase without ever needing to assume a particulate and therefore infective form in order to pass from one cell to another. It can be argued from this that the essential difference between a virus infection and a neoplasm is a difference in phase, and that if the factors responsible for controlling the development of the phases could be elucidated and suitably manipulated it would be possible to trans-form either type of disease into the other. Examples of viruses which produce benign proliferative lesions, and of neoplasms caused by viruses, are too well known to need further mention, and their existence emphasizes the fact that there is no sharp dividing line between the two types of disease. The formation of new protein in the cytoplasm which is characteristic of virus growth bears some resemblance to the events which precede mitosis in the normal cell, and suggests that there is something in common between virus multiplication on the one hand and cell division and neoplasia on the other. It has often been suggested that viruses resemble genes in their action, but it would be premature to support such a view until further evidence is available as to the means by which genes influence the activities of the cytoplasm. It is more helpful at the present time to compare viruses, not with genes alone, but with normal cell constituents in the widest sense, including also microsomes, plasmagenes, organizers and the unknown substances which probably pass out from the nucleus to control the activities of the cytoplasm. This comparison is made in the fourth column of Table 5, which needs little comment if the definition of cell constituent is borne in mind. It will be seen that the properties of viruses resemble those of cell constituents much more closely than those of bacteria, and it may be inferred from this that viruses are naturally related to normal cell constituents and may have originated from them. It would be unwise to be more dogmatic than this in the present state of knowledge, and virus workers would do well to frame their future work in accordance with this inference and thereby subject it to experimental proof.

REFERENCES

Bauer, D. J. (1947*a*). Xanthine oxidase and virus growth. *Nature*, **159**, 438.

Bauer, D. J. (1947*b*). Dehydrogenase activity in virus infection. *Brit. J. Exp. Path.*, **28**, 440.

Bauer, D. J. (1948). Association of xanthine oxidase with virus multiplication. *Nature*, **161**, 852.

Bauer, D. J. (1949). Multiplication of the animal viruses. *Nature*, **164**, 767.

Bauer, D. J. (1951*a*). Nucleic acid metabolism in virus infections. *Brit. J. Exp. Path.*, **32**. 7.

Bauer, D. J. (1951*b*). In preparation.

Breinl, F. & Glowazky, F. (1935). Über den Stoffwechsel des Vaccineerregers. *Klin. Wchnschr.*, **14**, 1149.

Brodie, M. & Wortis, S. B. (1934). Experimental poliomyelitis; cytologic studies of cerebrospinal fluid and respiratory metabolism of excised spinal cord and brain. *Arch. Neurol. & Psychiat.*, **32**, 1159.

Bronfenbrenner, J. J. & Reichert, P. (1926). Respiration of so-called filterable viruses. *Proc. Soc. Exp. Biol. Med.*, **24**, 176.

Caspersson, T. (1950). Outline of theory given in *The Biochemistry of the Nucleic Acids* by J. N. Davidson, Macmillan & Co. Ltd., London, 1950.

FAZEKAS DE ST. GROTH, S. (1948). Viropexis, the mechanism of influenza virus infection. *Nature*, **162,** 294.

FOSTER, C., JONES, J. H., HENLE, W. & DORFMAN, F. (1944). The effect of vitamin B_1 deficiency and of restricted food intake on the response of mice to the Lansing strain of poliomyelitis virus. *J. Exp. Med.*, **79,** 221.

GOLLAN, F. (1948). Effect of thyroxin on mouse susceptibility to polioencephalitis. *Proc. Soc. Exp. Biol. Med.* **67,** 362.

GREIFF, D., BLUMENTHAL, H. T. & PINKERTON, H. (1950). Influenza. III. Rapid alterations in the respiratory rate of embryonated eggs apparently caused by influenza virus toxin. *J. Exp. Med.* **91,** 335.

HALLAUER, C. (1951). Die Haemagglutination muriner Poliomyelitisvirusstämme. *Arch. ges. Virusforsch.*, **4,** 224.

HIRST, G. K. & PICKELS, E. G. (1942). A method for the titration of influenza haemagglutinin and influenza antibodies with the aid of a photoelectric densitometer. *J. Immunol.* **45,** 273.

HOAGLAND, C. L., WARD, S. M., SMADEL, J. W. & RIVERS, T. M. (1940). Biotin in elementary bodies of vaccinia. *Proc. Soc. Exp. Biol. Med.*, **45,** 669.

HOAGLAND, C. L., WARD, S. M., SMADEL, J. E. & RIVERS, T. M. (1941*a*). Constituents of elementary bodies of vaccinia. IV. Demonstration of copper in the purified virus. *J. Exp. Med.*, **74,** 69.

HOAGLAND, C. L., WARD, S. M., SMADEL, J. E. & RIVERS, T. M. (1941*b*). Constituents of elementary bodies of vaccinia. V. A flavin associated with the purified virus. *J. Exp. Med.*, **74,** 133.

HOAGLAND, C. L., WARD, S. M., SMADEL, J. E. & RIVERS, T. M. (1942). Constituents of elementary bodies of vaccinia. VI. Studies on the nature of the enzymes associated with the purified virus. *J. Exp. Med.*, **76,** 163.

HOLTMAN, F. (1946*a*). The effect of environmental temperature on mouse susceptibility to poliomyelitis virus. *Science*, **103,** 137.

HOLTMAN, D. F. (1946*b*). The effect of thiouracil and thyroactive substances on mouse susceptibility to poliomyelitis virus. *Science*, **104,** 50.

HOYLE, L. (1948). The growth cycle of influenza virus A. A study of the relations between virus, soluble antigen and host cell in fertile eggs inoculated with influenza virus. *Brit. J. Exp. Path.*, **29,** 390.

HOYLE, L. (1950). The multiplication of influenza viruses in the fertile egg. *J. Hyg.*, **48,** 277.

KALTER, S. S. (1949*a*). The effect of age upon susceptibility to infection with influenza virus. *J. Immunol.*, **63,** 17.

KALTER, S. S. (1949*b*). Host growth and its relation to influenzal virus infection. *J. Immunol.*, **63,** 29.

KALTER, S. S., SMOLIN, H. J., MCELHANEY, J. M. & TEPPERMAN, J. (1951). Endocrines and their relation to influenza virus infection. *J. Exp. Med.*, **93,** 529.

KALTER, S. S., STUART, D. C. & TEPPERMAN, J. (1950). Alterations in rate of influenza virus proliferation produced by growth hormone and testosterone. *Proc. Soc. Exp. Biol. Med.*, **74,** 605.

KUN, E. & SMITH, M. H. D. (1950). Effect of infectious myxoma virus on glycolysis of chorio-allantoic membrane of chick embryo. *Proc. Soc. Exp. Biol. Med.*, **73,** 628.

LITTLE, M. & LAWSON, R. B. (1951). The effect of FA and Lansing viruses on the cholinesterase activity of brain and spinal cord homogenates. *J. Immunol.*, **66,** 405.

MACFARLANE, M. G. & DOLBY, D. E. (1940). The enzymic activity of vaccinial elementary bodies. *Brit. J. Exp. Path.*, **21,** 219.

MACFARLANE, M. G. & SALAMAN, M. H. (1938). The enzymic activity of vaccinial elementary bodies. *Brit. J. Exp. Path.*, **19**, 184.

McLIMANS, W. F., SIEM, R. A., MARK, B. C. & PINSKA, E. (1950). A physiological study of virus parasitism. II. The effect of environmental temperature on the rates of oxygen consumption of normal eggs and eggs infected with Newcastle disease virus. *J. Immunol.*, **64**, 475.

McLIMANS, W. F., SIEM, R. A. & SCHOLLJEGERDES, V. R. (1950). A physiological study of virus parasitism. I. A method for determining the oxygen uptake of individual embryonated eggs. *J. Immunol.*, **64**, 463.

NICKLE, M. & KABAT, H. (1944). Specificity in the effects on brain metabolism of two differing neurotropic viruses. *J. Exp. Med.*, **80**, 247.

PARKER, R. F. & SMYTHE, C. V. (1937). Immunological and chemical investigations of vaccine virus. V. Metabolic studies of elementary bodies of vaccinia. *J. Exp. Med.*, **65**, 109.

PARODI, A. S., LAJMANOVICH, S., PENNIMPEDE, F. & MITTELMAN, N. (1948). Changes in embryonated eggs inoculated with influenza virus. *J. Immunol.*, **58**, 109.

PEARSON, A. E. & WINZLER, R. J. (1949). Oxidative and glycolytic metabolism of minced day-old mouse brain in relation to propagation of Theiler's GD VII virus. *J. Biol. Chem.*, **181**, 577.

PINKERTON, H., GREIFF, D., BLUMENTHAL, H. T. & HENSLEY, R. (1950). Influenza. II. The Effect of influenza virus multiplication on the oxygen consumption curves of embryonated eggs incubated at different temperatures. *J. Exp. Med.*, **91**, 331.

RACKER, E. & KABAT, H. (1942). The metabolism of the central nervous system in experimental poliomyelitis. *J. Exp. Med.*, **76**, 579.

RACKER, E. & KRIMSKY, I. (1946). Inhibition of phosphorylation of glucose in mouse brains by viruses and its prevention by preparations of diphosphopyridine nucleotide. *J. Exp. Med.*, **84**, 191.

RACKER, E. & KRIMSKY, I. (1947). Relation of iron salts to inhibition of glycolysis by Theiler FA virus of mouse encephalomyelitis. *J. Exp. Med.*, **85**, 715.

RASMUSSEN, A. F., WAISMANN, H. A., ELVEHJEM, C. A. & CLARK, P. F. (1943). Role of nutrition in response of host to poliomyelitis virus infection. (*a*) Influence of thiamin and riboflavin deficiencies on resistance to Theiler's virus and to Lansing strain poliomyelitis in mice. *J. Bact.*, **45**, 85.

SCHWARTZMAN, G. (1950). Enhancing effect of cortisone upon poliomyelitis infection (Strain MEF1) in Hamsters and Mice. *Proc. Soc. Exp. Biol. Med.*, **75**, 835.

SIEM, R. A., SMITH, B. C. & McLIMANS, W. F. (1950). Oxygen uptake of embryonated eggs infected with Western equine encephalitis virus. *Science*, **112**, 505.

STANLEY, W. M. (1945). The preparation and properties of influenza virus vaccines concentrated and purified by differential centrifugation. *J. Exp. Med.*, **81**, 193.

UTTER, M. F., REINER, J. M. & WOOD, H. G. (1945). Measurement of anaerobic glycolysis in brain as related to poliomyelitis. *J. Exp. Med.*, **82**, 217.

VICTOR, J. & HUANG, C. H. (1944). Depression of anaerobic glycolysis of embryonic tissue by Western strain of equine encephalomyelitis virus. Prevention of this effect by specific immune serum. *J. Exp. Med.*, **79**, 129.

WOOD, H. G., RUSOFF, I. I. & REINER, J. M. (1945). Anaerobic glycolysis of the brain in experimental poliomyelitis. *J. Exp. Med.*, **81**, 151.

WYCKOFF, R. W. G. (1951). Electron microscopy of chick embryo membrane infected with PR-8 influenza. *Nature*, **168**, 651.

*

DISCUSSION

N. W. PIRIE: This discussion, as discussions on viruses so often do, seems to have got bogged down in metaphysics. First: what is an enzyme? An enzyme is a substance that combines with its substrate in such a way that it catalyses an action and is then released in such a state that at least a proportion of it can go through the cycle again. Thus if it has only been shown to carry out the action once it is not an enzyme but it is not necessary for it to have been shown to carry it out hundreds of thousands of times as Dr Bauer suggests. Some enzymes do but not all of them need. Secondly: what is a particle? The ideas of Democritus, systematized by more recent authorities, made it likely that all substances were built up of atoms and so were particulate. The distinction that Bauer, Hoyle and some others in this Symposium make between particulate and non-particulate fractions is meaningless. The only significant question is 'How big are the particles'. This can be answered well enough if speakers explain what sort of centrifuge they use and I would like to urge Chairmen to ask everyone who gets involved in this particular piece of metaphysics to let us have this necessary information.

H. J. F. CAIRNS (and A. ISAACS): The influenza virus mucinase constitutes the most serious objection to Dr Bauer's hypothesis that viruses do not possess intrinsic enzymes and he has made Procrustean efforts to fit the experimental facts into his theory. There is a fundamental difference between agglutination by MM and influenza viruses, the former being a completely reversible reaction whereas the latter is not. Following elution from red cells the liberated influenza virus is able to agglutinate fresh red cells while the cells are no longer agglutinable by fresh virus. It was this ability of influenza virus to destroy red cells receptors repeatedly which first suggested to Hirst that the action was enzymic in nature.

Since then an overwhelming weight of evidence in favour of the enzymic nature of the influenza virus action has accumulated. Perhaps the most important part of this evidence is the work of Gottschalk and co-workers who have shown that following the interaction of purified influenza virus and electrophoretically pure urinary mucoprotein inhibitor, a dialysable split product can be separated from the reaction mixture (Gottschalk, *Nature*, 1951, **167**, 845). The split product is not formed when virus or mucoprotein are incubated alone, and there is no evidence of mucinase activity in uninfected tissues.

The alternative hypotheses which Dr Bauer has put forward would not explain satisfactorily why heated virus does not elute from normal red cells or unheated virus from periodate-treated red cells, or the action of virus on soluble inhibitors, which do not show Donnan equilibria. Evidence of the similar nature of the virus action on red cells and soluble inhibitors comes from the work of Bovarnick and de Burgh (*Science*, 1947, **105**, 550) and others who prepared soluble extracts from red cells which inhibit influenza virus agglutination, are inactivated on incubation with the virus, and which presumably contain the substrate of the enzyme action.

Finally, Gottschalk and Perry (*Brit. J. Exp. Path.*, 1951, **32,** 408) have shown that the enzymic activity of an infected allantoic fluid is intimately bound to its virus content since the ratio between the enzymic and agglutinating titres of a virus preparation remained constant when three different methods of purification of the virus were used. If it is true, as Dr Bauer has said, that alternatives to the enzymic theory have not been seriously considered this must be attributed to the weight of experimental evidence which renders alternative theories unnecessary.

D. J. BAUER: There is of course an important difference between the haemagglutination of influenza virus and that of the neurotropic viruses in that the red cell surface is altered in one case and not in the other, but this alteration is not in itself sufficient proof that an enzyme action has taken place. If the MM haemagglutination had been discovered first and the influenza haemagglutination some seven or eight years later I think we should be more inclined to attempt to explain the latter effect in terms of physical chemistry and would require more convincing proof than has yet been offered before accepting the enzyme theory. The work of Gottschalk and Perry quoted assumes the truth of the enzyme theory from the outset and merely shows that the property of haemagglutination is associated with the virus particle, a fact which nobody has ever disputed. The alternative hypotheses which I have put forward do not explain all the facts, but nor does the enzyme theory in my opinion. Facile assumptions based upon it may be fallacious, since the interpretation of the Francis effect as an enzyme action has now been shown to be incorrect by the work of Wilson Smith. The hypothesis of sub-unit dissociation easily explains the action of virus on soluble inhibitors, the prosthetic groups of which could be blocked by a relatively inexhaustible supply of sub-units, and it can be extended to explain the non-elution of heated virus without unduly stretching our credulity, since heating might well cause a structural alteration which prevents dissociation from occurring. However, I thought I had made it clear in my paper that I did not regard either of the hypotheses as the final answer, and I think that the complete explanation of the enzyme-like properties of influenza virus will eventually lie in something which we have not yet thought of.

The isolation of a product from the virus-substrate reaction could be convincing evidence of the enzyme nature of the reaction if the evidence were watertight. Gottschalk and Lind originally made their claim (*Nature*, 1949, **164,** 232) on the basis of experiments with ovomucin. This is not a pure substance, however, but contains an enzyme, lysozyme, which is known to liberate a substance which is nearly identical with the reaction product which they claim to have been liberated by the virus. In the absence of published details it is difficult to know if this objection has been adequately met by controls. The work was later repeated by Gottschalk (*Nature*, 1951, **167,** 845) with an electrophoretically homogeneous mucoprotein. More evidence may have been obtained than has actually been published, of course, but the results as far as they are disclosed in the latter paper admit of at least two other interpretations. Firstly, the virus might be the substrate and the mucoprotein the enzyme;

I am not suggesting that this is a likely explanation, but there is nothing in the paper which disagrees with it. Secondly, the reaction might be purely chemical and not enzymic. To prove the latter it would be necessary to show that the inhibitor is destroyed and the virus is not, and to equate the product formed with the amount of inhibitor disappearing. There would of course be practical difficulties in doing this, since the virus would break down to a certain extent when incubated, which would have to be allowed for, but the objection will have to be met before a rigid proof of enzyme activity can be offered. It would also be desirable to have a full treatment of the reaction in relation to the Michaelis-Menten equation. There is thus still much to be done in the way of proof, and in spite of the brisk and able rejoinder of Dr Cairns and Dr Isaacs I still feel that, in the words of Table 2, there is 'legitimate doubt as to whether this is an enzyme action'.

MARJORIE G. MACFARLANE: Dr Bauer has referred to the work on the enzymic activity of vaccinia virus carried out by Dr Salaman, Dr Dolby and myself, and by Dr Rivers and his colleagues at the Rockefeller Institute. I should like to contemplate this work, from a distance of twelve years or so, in a way which may be more constructive for an experimental approach than Dr Bauer's.

Firstly, the absence of oxidizing enzymes does not preclude the presence of protein-synthesizing enzymes in a virus; but the virus could probably only exercise its synthetic ability by stealing energy from the host. We pointed out at the time that a virus protein lying within a cell might be in as good a position to absorb this energy as protein-synthesizing enzymes of the cell. It is now possible to obtain suspensions of mitochondria, say from liver, which in a suitable medium will respire for hours and show the phenomenon of oxidative phosphorylation, i.e. adenylic acid added to the medium is phosphorylated to the high energy compound adenosinetriphosphate, which remains in solution in the medium; if yeast hexokinase, a foreign protein, is added together with its substrate glucose, the hexokinase utilizes the ATP for the synthesis of hexosemonophosphate. Taking this as a prototype for the stealing of energy it appears technically possible to construct a suitable system *in vitro* in which washed mitochondria are producing ATP, and to add to the medium firstly building blocks for protein synthesis and secondly other kinds of particles which have a presumptive claim to be able to synthesize proteins. One might begin with microsomes from a tissue which produces a characteristic protein recognizable by its activity, such as the thyroid or pancreas. Then it could be tested whether particular virus particles added to the medium with mitochondria alone could synthesize their characteristic protein—these would be the parasite-like group, resembling bacteria; or whether the synthesis of a particular virus required also the presence of microsomes which were 'infected' and changed their pattern of protein synthesis—this would be the 'controlling-influence' or gene-like group. There are of course other sub-cellular particles and combinations of cell constituents besides microsomes whose protein-synthesizing capacity could be tested. There is no point in elaborating this idea; I merely wish to point out that while even five years ago it was hardly possible to get a cell-free but respiring system suitable for testing the theories so long put forward, to-day it seems feasible, thanks primarily to the work of Bensley and of Claud and latterly of many other workers.

Considering the only positive result we obtained, I wish first to mention an additional experiment on the phosphatase activity of vaccinia virus. Dr A. S. McFarlane, of the National Institute for Medical Research, some years ago obtained some remarkably homogeneous preparations of vaccinia for electron microscope studies, by treating suspensions of elementary bodies with *M* Na Cl. Through his kindness I was able to test samples before and after this treatment. The purified virus showed phosphatase activity of the same level as the usual preparation:

Preparation		μg. phenol/hr./mg. liberated from phenylphosphate at 37°
V41	Before treatment	55
	After treatment	45
V46	After treatment	86
Dermal pulp residues		60–120
A purified bone phosphatase		1100

Rivers and his colleagues thought it likely, though by no means proven, that all the phosphatase present was adsorbed, i.e. was of host origin. This may be so; there is no evidence on this, and in a sense the origin is irrelevant. The interesting thing is that phosphatase is adsorbed rather specifically and held rather firmly. Dr Bauer has to face the experimental fact that vaccinia virus of high purity has not been obtained free from phosphatase. There is no *a priori* reason that adsorption should take place only during the preparation; as the virus has demonstrably the capacity to adsorb this enzyme it must be assumed that it can do this *in vivo.*

It is typical of a parasite to get what it needs by hook or by crook with the least expenditure of energy. The virus can get a phosphatase by hook; can it use it for some crooked end? We pointed out previously that the presence of an enzyme capable of hydrolysing adenylic acid may be a primary cause of degeneration in an infected cell, a necessary accessory in providing an environment in which the act of reproduction can occur. It seems a pity to discount completely properties, even stolen properties, which may give a slender clue to the mechanism of synthesis.

D. J. BAUER: It would be interesting to see if new virus protein could appear in association with a suspension of mitochondria, but the scheme proposed differs little from the unavailing attempts which have often been made to propagate viruses in tissue homogenates in the absence of intact cells. The difficulty would also arise that an enzyme could still carry out its ordinary activities whether it was adsorbed on to a virus or on a completely inert particle, as for example dead virus, and one could not therefore safely infer that activities observed *in vitro* were any sort of counterpart of what takes place inside the cell.

The unfortunate fact that vaccinia virus adsorbs phosphatase from solution makes it impossible to determine whether this activity is an intrinsic property of the virus or not. I have incidentally observed that the alkaline phosphatase activity of mouse brain is reduced in infection with a mouse-adapted strain of vaccinia, but this has little bearing on the present problem. I have already suggested (*Nature*, 1949, **164,** 767) that a virus which presumably has to establish contact with the enzymes of the host cell might be expected to have an affinity for these enzymes, and the adsorption of phosphatase might merely be a somewhat irrelevant expression of this property. The phosphatase could even represent a host component which has become caught up in the virus body without representing an essential part of its structure.

L. HOYLE: I had always regarded the influenza haemagglutinin as an enzyme especially in view of the extensive work on the subject by the Melbourne School. One of the problems a virus has to solve is that of entering and leaving cells, and an extremely efficient way of achieving this is the manufacture of an enzyme attacking some constituent of the cell wall.

D. J. BAUER: I have never been satisfied that a virus needs to leave an infected cell. If it represents a perverted cell constituent it would not need to enter another cell in order to complete a life cycle or even to maintain itself. The points made by Bawden and Pirie in their paper in this Symposium are also applicable to animal viruses, and it may be that an animal virus represents, out of many abnormal nucleoproteins, the one which possesses the chance property of infectivity. Dr Hoyle himself has in any case clearly shown that a normal cell of the chorioallantoic membrane will cast off protoplasmic processes when placed in an unfavourable environment, and this seems a sufficiently adequate explanation for the liberation of influenza virus, or indeed of any virus whether haemagglutinating or not, without postulating the possession of an enzyme activity.

A. KLECZKOWSKI: The fact that viruses do not show any of the few enzymic activities for which they were tested, seems quite insufficient for a conclusion that they are devoid of any enzymic activity. It is quite possible that virus particles can catalyse some reactions intimately connected with virus synthesis, and such reactions may not be any of the well-known reactions which were not found to be catalyzed by viruses.

It cannot be assumed that the mechanism of virus multiplication relies entirely on enzymes of the host cell, for in the absence of the virus these enzymes do not produce the virus. The virus must be there to cause these enzymes to do something they cannot do by themselves. Whether or not the process of modification or reorganization of enzymes or of their activities is to be considered an enzymatic or a ‘ co-enzymatic ’ process, would simply depend on the definition of the words.

D. J. BAUER: It is of course easy to postulate that viruses possess enzyme activities that have not yet been invented, but I have only been concerned with assessing the evidence available at the present time. The statement that the virus cannot rely entirely on the host enzymes since these enzymes do not make virus in an uninfected cell is an obvious *non sequitur*. The following statement that 'the virus must be there to cause these enzymes to do something they cannot do by themselves' is merely a re-statement of my own views in practically the same words.

NUCLEIC ACIDS IN VIRUS MULTIPLICATION

ROY MARKHAM

Agricultural Research Council's Plant Virus Research Unit, Cambridge

INTRODUCTION

Although viruses were first recognized as such in the latter half of the last century very little was known about their real nature until 1933, when M. Schlesinger managed, by a method of combined ultrafiltration and high-speed centrifuging, to isolate one of the large bacteriophages (WLL) affecting *Escherichia coli.* In the next year (Schlesinger, 1934) he showed that his preparations contained protein and phosphorus, and gave reactions for carbohydrate, and he suggested that his bacteriophage might be a nucleoprotein. Finally, in 1936, he demonstrated that both this 'phage' and a staphylococcal phage gave a Feulgen reaction, i.e. contained deoxyribonucleic acid.

At about this time Bawden, Pirie, Bernal & Fankuchen (1936) reported that the virus of tobacco mosaic, which had been isolated in the previous year by Stanley (1935) was in fact a nucleoprotein of the ribose type and in the following years Bawden and Pirie (1937, 1938) demonstrated that a number of plant viruses were ribonucleoprotein in nature. Their observations were at first challenged, but soon were widely accepted with the result that for some years all viruses regardless of size and complexity were referred to as nucleoproteins.

Amongst the animal viruses several have been reported to contain deoxyribonucleic acid only, for example, vaccinia virus (Hoagland, Lavin, Smadel & Rivers, 1940), while others have been reported to contain ribonucleic acid only, for example, equine encephalomyelitis (Sharp, Taylor, Beard, Finkelstein & Beard, 1940) and 'poliomyelitis' virus (Gard, 1943), and yet others like influenza have been reported to have both (Knight, 1947*a*). It seems probable that some of these results will have to be revised in the light of later work, but there is now substantial unanimity among workers in the field that all viruses contain both protein and nucleic acid. This is of some interest in view of the importance which cytologists and others attach to nucleic acid, both from a genetic point of view and from the point of view of protein synthesis. Both these properties are, of course, intimately associated with viruses, as, however much they may be parasitic upon the host cell's synthesizing systems, they must themselves carry some mechanism for directing the latter into the correct paths, and they must also have a structure or structures controlling the 'genetic' composition of the particles formed. In the case of several plant viruses the particles are known to be very uniform in their structure, as uniform in fact as many purified proteins (and more uniform than most proteins) and they only contain protein and nucleic acid, so it seems fairly certain that a study of the composition and relationship of these two constituents will throw light upon the mechanism of the reproduction of plant viruses.

THE PLANT VIRUS NUCLEOPROTEINS

The plant viruses which have been purified show considerable variations in their general properties. Some, like potato virus X and tobacco mosaic virus, consist of greatly elongated particles, which probably have some repeat structure in their length, while others are approximately spherical. The latter, in general, contain more nucleic acid than do the rod-shaped forms. A table of the general composition of some of the plant viruses which have been purified and, in the case of the spherical ones, crystallized is given in Table 1.

Table 1. *The nucleic acid content of some plant viruses.*

Virus	% ribonucleic acid
Tobacco mosaic and strains[1]	6
Tomato bushy stunt[2]	16
Tobacco necrosis[3]	17
Southern bean mosaic[4]	21
Potato X[5]	6
Turnip yellow mosaic[6]	35

References

[1] Bawden & Pirie (1937).
[2] Bawden & Pirie (1938*a*).
[3] Pirie *et al.* (1938).
[4] Miller & Price (1946).
[5] Bawden & Pirie (1938*b*).
[6] Markham (1951).

Until very recently all ribonucleic acids, with the exception of that from pancreas (which in any case is an artefact of preparation) were regarded as having very much the same composition, largely because there were no really suitable methods available for the analysis of these substances, although it is of interest to note here that Bawden & Pirie (1937) commented upon an apparent physical difference between the nucleic acid which they isolated from strains of tobacco mosaic virus and the material available commercially as 'yeast nucleic acid'. Consequently much more work has been done on the composition of the protein components of plant viruses, as although the methods available were in many cases not more than semi-quantitative by modern standards, this approach was possible.

Some very interesting results have come out of the analyses of the proteins of plant viruses. Perhaps the most striking one is the fact that highly basic proteins of the protamine or histone type, which are associated with nucleic acids in such cells as animal spermatozoa, are not to be found in the plant viruses. The protein components of the plant viruses are in fact rather acidic. The way in which the nucleic acid is joined to such proteins is unknown, but it seems that the old idea of a salt linkage between strongly basic protein and nucleic acid is by no means certain, though of course a number of basic amino acids are to be found in any protein.

It has also been found (Knight, 1947*b*) that there can be a considerable variation in the general composition of the protein components of related plant viruses. Those that have been studied most have been the strains of the tobacco mosaic virus group. All of these viruses are similar morphologically and in their gross chemical composition, but may be differentiated by the use of serological techniques into a number of strains. A certain amount of differentiation may also be made by the study of the host symptomatology, but this may be very misleading, because viruses giving identical symptoms on plant may sometimes be readily distinguished by serological methods, while two viruses which cannot be differentiated from each other by serological or by chemical techniques, may differ to such an extent in their virulence that one kills its host outright while the other produces only mild symptoms.

The most striking chemical differences in the proteins of the tobacco mosaic group are the presence or absence of individual amino acids in strains. Thus histidine which is not found in the 'type' strain is to be found in the 'rib-grass' virus of Holmes (1941) to the extent of 0·72% (Knight, 1947*b*), while the viruses known as Cucumber viruses 3 and 4 have no sulphur-containing amino acids at all (Knight, 1949). These and lesser differences in composition are apparently 'hereditary', that is to say they are characteristic of individual strains, and are not influenced by such factors as the species of host plant. For example the rib-grass virus and the common tobacco mosaic virus maintain their individual amino acid compositions when grown on such different hosts as Phlox and tobacco (Gaw & Stanley, 1947). The report of a significant difference in composition in a strain of tobacco mosaic virus found after two successive mutations (Knight, 1947*b*) must be interpreted with some caution, as, apart from the difference being slight it is exceedingly difficult to control an experiment of this type adequately, and to ensure that the virus was not a mixture either at the beginning of the experiment or at some intermediate stage.

THE CASE OF THE TURNIP YELLOW MOSAIC VIRUS

The role of the protein constituent of plant viruses is undoubtedly very important, but there is some evidence that the nucleic acid is in fact the substance directly controlling virus multiplication. Some years ago Kenneth Smith and I (Markham & K. M. Smith, 1949) isolated and crystallized a new plant virus from turnip, which we have called the turnip yellow mosaic virus, and this virus has turned out to have some most unexpected properties.

When we first isolated turnip yellow mosaic virus it crystallized (Fig. 1) as regular octahedral crystals having a fairly uniform composition, but one which varied just so much more than our analytical technique could detect, that we were in despair of getting accurate analyses. Eventually we made a large batch of crystals and spent several weeks analysing them and getting satisfactory replicate analyses. We also did several Tiselius electrophoresis runs and found, as we expected, that the substance was homogeneous (Fig. 2) at several pH values, the sharpness of the electrophoresis peaks, due to the low diffusion constant of the virus, being such that we should have noticed the presence of quantities of a few per cent

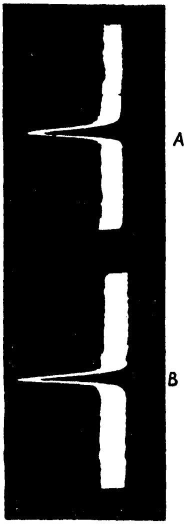

Fig. 2. Electrophoresis pattern of a 0·5% solution of turnip yellow mosaic virus. A, descending, and B, ascending boundaries respectively. pH 5·38.

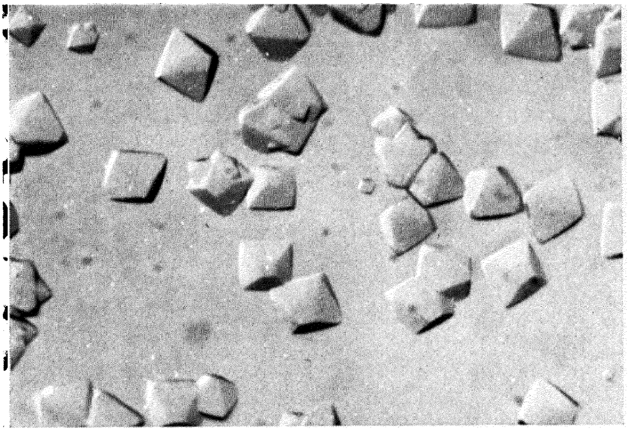

Fig. 1. Crystals of the turnip yellow mosaic virus. × 100.

To face p. 88

of material having an electrophoretic mobility differing by more than about 5%. It was therefore with some surprise that we found that when centrifuged on an ultracentrifuge (by Drs Cecil and Ogston), there were two quite distinct components sedimenting at different rates (about 2:1) (Fig. 3). This meant that, if in fact there was only one kind of substance present, about 20–30% of it would have had to have dissociated into quarters. This was very surprising, and in our view unlikely as there was no indication of any inhomogeneity in the electron micrographs which we had obtained. Eventually by means of high speed centrifuging, we managed to obtain a quantity of the slower sedimenting or 'top' component for analysis, and found that when it was free of the other component it contained neither phosphorus nor sugar: in fact it was not a nucleoprotein at all. Extensive serological experiments carried out by us and by our colleague Dr R. E. F. Matthews, however, failed to detect any difference in the proteins of the 'top' or the 'bottom' component, while the two substances crystallized identically, with the differences that one could distinguish one type of crystal from the other in shortwave ultraviolet light because of the absence of nucleic acid in the 'top' component crystals, and, because of the lower density of the latter, we could separate an *artificial* mixture of the crystals by centrifuging then in a sodium thiosulphate solution having the correct density. As the particles themselves form mixed crystals quite readily, it is unfortunately impossible to use this simple technique for separating the mixture.

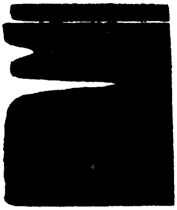

Fig. 3. A solution of turnip yellow mosaic virus protein in the ultracentrifuge showing the two components. 24,000 r.p.m. pH 7·0.

We have made a number of measurements of the size of these two sets of particles by different methods, and other workers have also made similar measurements (e.g. Bernal & Carlisle, 1948, 1951, by X-ray diffraction techniques) and they all confirm that the particles are either identical in size and shape, or the nucleoprotein, or 'bottom', component particles are in fact slightly smaller, for some reason concerned with the unusually large amount of nucleic acid in the particles.

A very interesting thing arising from this piece of work is that the 'top' component particles, besides being rather poor antigens, as far as the stimulation of antibody formation in rabbits is concerned, are non-infectious. Now the failure of viruses to infect after they have been treated in various ways is well known, but no obvious chemical change has been shown in these cases (for example tomato bushy stunt virus heated to temperatures of 20°–50°, for periods of weeks at the lower temperatures, and of minutes at the higher ones), but in this case it is possible by purely physical methods to isolate non-infectious particles from infectious ones. At first we were uncertain as to whether the top component might not be an artefact produced in our isolation procedure, which, incidentally, uses a concentration of alcohol (22%) not far below that at which the virus itself is denatured and the nucleic acid liberated, i.e. 33% by volume. We managed to show that the 'top' component was not an artefact, by the simple procedure of putting a quantity of pure 'bottom' component into minced leaf tissue from healthy plants and treating it just as we would treat diseased plant material. The virus was isolated with ease and when ultracentrifuged was found to consist only of 'bottom' component (Fig. 4). This experiment shows that none of the steps in the purification procedure leads to the loss of nucleic acid from the nucleoprotein, and, incidentally, we have tried to liberate the nucleic acid from the latter by various methods, so far without success.

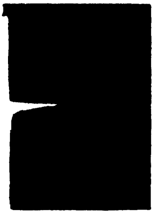

Fig. 4. Pure 'bottom' component of turnip yellow mosaic virus on the ultracentrifuge. 24,000 r.p.m., pH 9·0.

Over the last few years we have made a large number of preparations of turnip yellow mosaic virus. Over the period of twelve months we made fourteen large scale preparations, amounting to a total of over thirty grams dry weight of crystalline material, and each preparation has been subjected to analysis, while several have been examined on the ultracentrifuge as well. From the ratio of P/N in our material it is a simple matter to calculate the percentage of the two components, as the P content of the pure nucleoprotein has been found to be 3·43%, while the nucleic acid itself has a P content of 9·79% (calculated from its known composition, Markham & Smith, 1951*a*) giving a nucleic acid content of the virus nucleoprotein of 35%. This value is considerably higher than the earlier values which we published, which were estimated from the average P content of several preparations and the amount of the two components assessed from the ultracentrifuge diagram. It was not in fact for several years that we were able to isolate the 'bottom' component in a pure state. Oddly enough the method which we employ is essentially that which is used in the routine purification of viruses in many laboratories, expecially in the U.S.A., i.e. differential centrifugation. If this process is carried out in rather dilute solutions in an air-driven vacuum centrifuge, or in stronger solution (2% or more total weight) in high-speed angle centrifuges (about 10–15,000 r.p.m.), we have found that the recovery of the lighter component is *much* smaller than one would expect from simple sedimentation theory. This can be explained quite easily using a more complex theory of sedimentation (Markham, 1951) which may have uses in other problems, but the nett effect is that after a small number of centrifugation-cycles *all* the 'top' component is lost in the supernatant fluids. This fact is of considerable interest as it shows that we might never have noticed the presence of more than one substance in plants diseased with this virus if we had used the methods which are now conventional, rather than the salt precipitation methods which are more convenient in the case of this and several other viruses. It is therefore quite likely that such a phenomenon will be overlooked if it occurs in other viruses, and I see no reason why one should think this case exceptional.

The results of our analyses show that in all cases infection with the nucleoprotein is inevitably followed by the production of some 'top' component, usually between 20 and 35% by weight of the total protein material, consisting of the latter or, in terms of numbers of particles, about 40% of the ones produced are not capable of further multiplication. The reason for the production of this large number of sterile particles is obscure at the moment. It would be nice to think that they were a stage in the multiplication process of the virus, but this does not seem very likely, as there is little correlation between the proportion of the two components and the length of infection. Similarly, they do not seem to be breakdown products of the virus, while in either case the mechanical difficulty of inserting very asymmetrical material like nucleic acid into these particles or removing it would appear to present great difficulties, because we have shown that it is unlikely that the nucleic acid is on the surface of the particles (Markham, 1951), merely from the fact that there is no room for it there, as well as for other good reasons, such as the quantity of antibody bound by the two kinds of particles. A plausible explanation, but one for which we have no proof, is that the host cells' mechanism for nucleic acid synthesis is unable to keep pace with its virus protein synthesizing ability. This argument is not obvious until one realizes that during the production of this virus the host plant has to provide enough material to make up to 400 μg. of nucleic acid for each ml. of sap volume, a quantity which is not approached by any other known plant virus, although the protein produced is rather less than in a tobacco mosaic virus infection. (These figures are for the virus liberated in the sap on mincing, and do not include virus bound by insoluble cell constituents.)

Whatever the explanation for the presence of the 'top' component, it seems certain that the nucleic acid plays some very important part in the multiplication process, and it is largely for this reason that we have concentrated so much on trying to elucidate the structure of nucleic acids, particularly those of viruses.

THE NUCLEIC ACIDS OF SOME PLANT VIRUSES

Until the last few years there have been practically no good methods for the analysis of nucleic acids, but recently the paper chromatographic technique has been applied successfully to this problem (Hotchkiss, 1948; Vischer & Chargaff, 1948; Smith & Markham, 1950), and by means of the more refined techniques (Wyatt, 1950; Markham & Smith, 1951*a*) it is possible to make accurate analysis of any nucleic acid, and to obtain its general nucleotide composition.

For many years all nucleic acids were thought to consist of two types, those containing ribose, or the ribonucleic acids (I do not intend to use the term pentose nucleic acids, as there is now good evidence that the sugar must be ribose), and those containing deoxyribose, and that, with the possible exception of the ribonucleic acid of pancreas, all ribonucleic acids were identical, and all deoxyribonucleic acids were identical. As soon as adequate analysis was made, this tacit assumption was shown to be untrue, and it was evident that there were great variations in the possible structure, and even (Wyatt, 1950) that there were constituents that had not been detected previously. The pyrimidine, 5-methyl cytosine, which Wyatt discovered in our laboratory to be a constituent of many deoxyribonucleic acids, is present in some of the latter in such small amounts that it seems unlikely that it could be a constituent of more than a small proportion of the molecules, and this suggested to us that the nucleic acids are in fact mixtures of similar but not identical substances. This being so, one might well expect that the viruses would exhibit very large variations in their nucleic acids, as the tendency to average out the general composition of the nucleic acids will obviously be a function of the quantity of the latter per cell or per particle. In general this has proved to be true, and we have so far been able to analyse fourteen plant virus nucleic acids, five from tobacco mosaic virus strains, seven from strains of potato virus *X*, the tomato busy stunt virus nucleic acid, and that from the turnip yellow mosaic virus. These analyses have shown two main things: first that related viruses have very similar nucleic acids, and secondly that the difference between unrelated viruses is reflected in the composition of their nucleic acids, to such an extent that one could almost use analyses of the nucleic acids as a diagnostic character. Although the ribonucleic acids of the host plants have not yet been analysed, the experiments so far made provide their own internal control. In Fig. 5 the composition of seven plant virus nucleic acids is plotted, the areas of the sectors being proportional to the nucleotide compositions of the individual substances. Reading from 12 o'clock clockwise the sectors are: guanylic acid (black), adenylic acid (white), cytidylic acid (black), and uridylic acid (white). The nucleic acids are from: (1) and (2), two distantly related strains of potato virus *X* (B and XL) (Rønde-Kristensen & Markham, unpublished), (3), (4) and (5), three strains of tobacco mosaic virus (tomato mosaic, cucumber 4, and type tobacco mosaic) (Markham & Smith, 1950), (6) tomato bushy stunt virus, (7) turnip yellow mosaic virus (Markham & Smith, 1951*a*) and (8) a typical yeast nucleic acid for comparison (Smith & Markham, 1950). The host plants used were (1) and (2) tobacco, (3) tomato, (4) cucumber, (5) tobacco, (6) Datura and (7) Chinese cabbage.

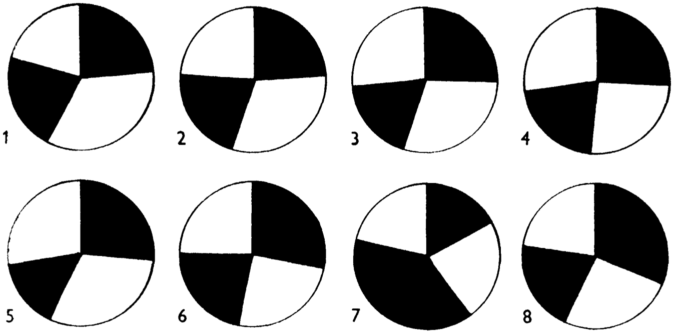

Fig. 5. The nucleotide composition of the ribonucleic acids of (1) Potato virus *B*, (2) Potato virus XL, (3) tomato mosaic virus, (4) cucumber virus 4, (5) tobacco mosaic virus, (6) tomato bushy stunt virus, (7) turnip yellow mosaic virus, and (8) a typical yeast nucleic acid. The sectors, which are proportional in area to the molar ratios of nucleotides, are from 12 o'clock clockwise guanylic acid (black), adenylic acid (white), cytidylic acid (black) and uridylic acid (white).

We have also carried out analyses of a different type on some ribonucleic acids (Markham & Smith, 1951*b* and unpublished), by digesting the nucleic acids with enzymes and isolating the fragments. These experiments have shown that the ribonucleic acids are all built up in a similar way, and the large variety of the constituents, combined with the presence of what are evidently terminal groups of the molecules, has confirmed our earlier suspicion that these nucleic acids are mixtures. When one considers that the amount of nucleic acid in a virus such as that of turnip yellow mosaic is equivalent to a molecular weight of two million, it is evident that there is considerable scope for variation among the nucleic acids. In this virus one can estimate approximately from the data at our disposal (which includes the probability that the virus particle is composed of four identical subunits), that there may be as many as twenty to thirty different kinds of nucleic acid chain in each molecule.

Summing up it seems that the fundamental differences between groups of plant viruses are correlated with their nucleic acid composition, whereas 'strain' differences are correlated with the amino acid composition rather than with that of the nucleic acid. The nucleic acid also appears to play a leading part in the ability of a virus to multiply, whereas the pattern provided by the protein of a virus would appear to be inadequate in itself to ensure its reproduction.

REFERENCES

Bawden, F. C. & Pirie, N. W. (1937). The isolation and some properties of liquid crystalline substances from solanaceous plants infected with three strains of tobacco mosaic virus. *Proc. Roy. Soc.*, **123,** 274.

Bawden, F. C. & Pirie, N. W. (1938*a*). Crystalline preparation of tomato bushy stunt virus. *Brit. J. Exp. Path.*, **19,** 251.

Bawden, F. C. & Pirie, N. W. (1938*b*). Liquid crystalline preparations of potato virus ' X '. *Brit. J. Exp. Path.*, **19,** 66.

Bawden, F. C., Pirie, N. W., Bernal, J. D. & Fankuchen, I. (1936). Liquid crystalline substances from virus infected plants. *Nature*, **138,** 1051.

Bernal, J. D. & Carlisle, C. H. (1948). Unit cell measurements of wet and dry crystalline turnip yellow mosaic virus. *Nature*, **162,** 139.

Bernal, J. D. & Carlisle, C. H. (1951). The place of X-ray diffraction methods in the estimation of the size and mutual arrangements of colloidal particles. *Faraday Soc. Discussion*, **11,** 227.

Gard, S. (1943). Thesis, Upsala. Purification of the poliomyelitis viruses.

Gaw, H. Z. & Stanley, W. M. (1947). Comparative properties of purified preparations of two distinctive strains of tobacco mosaic virus obtained from diseased Turkish tobacco and Phlox plants. *J. Biol. Chem.*, **167,** 765.

Hoagland, C. L., Lavin, G. I., Smadel, J. E. & Rivers, T. M. (1940). Constituents of elementary bodies of vaccinia. I. Certain basic analyses and observations on lipid components of the virus. *J. Exp. Med.*, **71,** 737.

Holmes, F. O. (1941). A distinctive strain of tobacco mosaic virus from *Plantago*. *Phytopathology*, **31,** 1089.

Hotchkiss, R. D. (1948). The quantitative separation of purines, pyrimidines and nucleosides by paper chromatography. *J. Biol. Chem.*, **175,** 315.

KNIGHT, C. A. (1942). Basic amino acids in strains of tobacco mosaic virus. *J. Amer. Chem. Soc.*, **64,** 2734.

KNIGHT, C. A. (1947*a*). The nucleic acid and carbohydrate of influenza virus. *J. Exp. Med.*, **85,** 99.

KNIGHT, C. A. (1947*b*). The nature of some of the chemical differences among strains of tobacco mosaic virus. *J. Biol. Chem.*, **171,** 297.

KNIGHT, C. A. (1949). The apparent sulfur in cucumber virus 4. *J. Amer. Chem. Soc.*, **71,** 3108.

MARKHAM, R. (1951). Physicochemical studies of the turnip yellow mosaic virus. *Faraday Soc. Discussion*, **11,** 221.

MARKHAM, R. & SMITH, J. D. (1949). Chromatographic studies of nucleic acids. 1. A technique for the identification and estimation of purine and pyrimidine bases, nucleosides and related substances. *Biochem. J.*, **45,** 294.

MARKHAM, R. & SMITH, J. D. (1950). Chromatographic studies of nucleic acids. 3. The nucleic acids of five strains of tobacco mosaic virus. *Biochem. J.*, **46,** 513.

MARKHAM, R. & SMITH, J. D. (1951*a*). Chromatographic studies of nucleic acids. 4. The nucleic acid of the turnip yellow mosaic virus, including a note on the nucleic acid of the tomato bushy stunt virus. *Biochem. J.*, **49,** 401.

MARKHAM, R. & SMITH, J. D. (1951*b*). Structure of ribonucleic acid. *Nature*, **168,** 406.

MARKHAM, R. & SMITH, K. M. (1949). Studies on the virus of turnip yellow mosaic. *Parasitology*, **39,** 330.

MILLER, G. L. & PRICE, W. C. (1946). Physical and chemical studies on southern bean mosaic virus. III. Electrophoretic and nucleic acid studies. *Arch. Biochem.*, **11,** 337.

PIRIE, N. W., SMITH, K. M., SPOONER, E. T. C. & MCCLEMENT, W. D. (1938). Purified preparations of tobacco necrosis virus (*Nicotiana* virus 11). *Parasitology*, **30,** 543.

SCHLESINGER, M. (1933). Reindarstellung eines Bakteriophagen in mit freiem Auge sichtbaren Mengen. *Biochem. Z.*, **264,** 6.

SCHLESINGER, M. (1934). Zur Frage der chemischen Zusammensetzung des Bakteriophagen. *Biochem. Z.*, **273,** 306.

SCHLESINGER, M. (1936). The Feulgen reaction of the bacteriophage substance. *Nature*, **138,** 508.

SHARP, D. G., TAYLOR, A. R., BEARD, D., FINKELSTEIN, H. & BEARD, J. W. (1940). Properties of the isolated equine encephalomyelitis virus (eastern strain). *Science*, **92,** 359.

SMITH, J. D. & MARKHAM, R. (1950). Chromatographic studies of nucleic acids. 2. The quantitative analysis of ribonucleic acids. *Biochem. J.*, **46,** 509.

STANLEY, W. M. (1935). Isolation of a crystalline protein possessing the properties of tobacco mosaic virus. *Science*, **81,** 644.

VISCHER, E. & CHARGAFF, E. (1948). The composition of the pentose nucleic acids of yeast and pancreas. *J. Biol. Chem.*, **176,** 715.

WYATT, G. R. (1950). Occurrence of 5-methyl-cytosine in nucleic acids. *Nature*, **166,** 237.

WYATT, G. R. (1951). The purine and pyrimidine content of deoxypentose nucleic acids. *Biochem. J.*, **48,** 584.

DISCUSSION

F. C. BAWDEN: Would Dr Markham say a little more about his theory of sedimentation to explain the separation of the 'bottom' and 'top' components? When Pirie and I were separating fractions of tobacco mosaic virus particles of different sizes and activities, we got unexpectedly large differences between material that was sedimented and that which remained in the supernatant fluid. Dr Markham's theory may help to explain this sharp separation and also the fact that those who have worked only with sedimented material describe preparations that are more uniform in particle size than ours.

Has Dr Markham measured the ratio of 'top' and 'bottom' components at different intervals after infection? If his suggestion is true that the 'top' component lacks nucleic acid because the host cell is unable to produce enough nucleic acid, then the proportion of 'top' component would be expected to increase as the total production of anomalous protein increases. If the experiments have not yet been done, I am prepared to postulate the answer and suggest that this will not happen, but that the two components will be found in more or less constant proportions regardless of the total quantity of anomalous protein extracted.

R. MARKHAM: The theory of sedimentation of particles in preparative centrifuges is rather complex, and it is probably impossible to formulate a complete mathematical treatment of such centrifugation. It is, however, possible to take some simple cases and deduce what should happen. It is usually assumed that the rate of sedimentation of particles in centrifuges can be calculated from the Svedberg expression: $s = dx/dt/\omega^2x$, where s is the sedimentation constant, x is the radius, t the time and ω the angular velocity. From this, it will be seen that the rate of deposition of particles from solution is approximately linear (the value of x being large compared with the distance from the meniscus of the tube to the bottom). This expression, however, assumes that all the particles sediment in a regular, even manner and are not disturbed in their motion by currents in the bulk of the liquid due to mechanical vibrations and thermal convection, and consequently that a boundary is formed at the meniscus and travels towards the bottom of the tube. This boundary, however, is only stable if the density gradient which it represents is sufficiently great to prevent movement in the liquid from disrupting it. If the solution is very weak, or if the thermal and mechanical disturbances are large, a boundary is no longer able to form. In this case the rate of sedimentation is given by the expression: $Ct/Co = e^{-rKAt/V}$, where Ct is the concentration of solute at time t and Co the original concentration, r is the radius at the *bottom* of the tube, A the area onto which sedimentation is taking place and V the total liquid volume. $K = \omega^2 s$. Now it happens that with the ordinary dimensions of centrifuges, the sedimentation in the second case is *much* slower than in the first case. It also happens that at certain concentrations of two or more different substances, both types of sedimentation can proceed simultaneously, and in the case of turnip yellow mosaic virus solution, this is achieved at a concentration of about 1–4 per cent. total protein per ml. in electrically driven angle centrifuges running at 12,000 to 16,000 r.p.m., and in vacuum ultracentrifuges at a much lower concentration. Consequently by choosing the correct concentration it is possible to effect separations which at first sight would appear to be impossible. If sedimentation is repeated the concentration used is, of course, less critical. It is also possible by the addition of an extra substance having a suitable value of *s*, to take advantage of this phenomenon and use the added substance as a liquid 'ultrafilter' membrane. Reference may be made to Bechhold & Schlesinger (*Biochem. Z.*, 1931, **236**, 386) and to me (*Faraday Soc. Discussion*, 1951, **11**, 221).

In reply to Mr Bawden's second question, there is apparently no correlation between the ratio of 'top' component to 'bottom' component with time of infection, or for that matter with any other factor. (The ratio does of course vary a little from preparation to preparation.) Although I am now inclined to agree that the production of 'top' component may not be due to lack of synthetic ability in the plant, it seems to me that the evidence does not enable one to decide definitely one way or the other, as we cannot follow the virus multiplication in individual cells, but only in whole leaves.

N. W. PIRIE: The assumption that a grouping that is found inside a virus is not attached to the main virus structure, even supposing that the evidence for this disposition is unequivocal, does not seem to be necessary. For example my stomach seems to me to be more firmly attached to me than my jacket. It seems also that Markham should have used the plural when speaking of 'The *way* in which the nucleic acid is joined to such proteins'. Urea splits nucleic acid off tobacco mosaic virus and potato virus X but, although it inactivates and denatures the viruses, it does not split nucleic acid off tomato bushy stunt and tobacco necrosis virus (Bawden & Pirie, *Biochem. J.*, 1940, **34**, 1258). Furthermore strontium nitrate splits the nucleic acid from tobacco mosaic virus but not from bushy stunt or tobacco necrosis viruses.

R. MARKHAM: I should imagine that Pirie's stomach not only seems, but actually is, more firmly attached to him than his jacket, although I have heard of people having some trouble with zip fasteners. What I wished to imply in my paper was that there is no good reason for invoking linkages of the covalent type to explain the nucleic acid-protein relationship in many plant viruses, as in most cases one could explain the observed phenomena by means of the steric arrangement of the two components. Naturally, as Kossel (*Archiv Anat. Physiol.*, 1893, p. 157) observed, if proteins and nucleic acids occur together it would be surprising if they were not combined in some way, but this way (or ways) probably involves rather unstable bonds.

J. S. K. BOYD: Dr Markham has stated that the 'top' component of turnip yellow mosaic virus is non-infectious whereas the 'bottom' component is. Is it possible to expose susceptible plants first to 'top' component and subsequently to 'bottom' component, and if so does the former block the action of the latter?

R. MARKHAM: We have done experiments of the type suggested and we are under the impression that the 'top' component is slightly inhibitory. Unfortunately the experimental difficulties are such that we have spent little time on this problem. One of the major factors is that there is some uncertainty that any batch of plants will produce local lesions adequate for assay, while the production of 'pure' 'top' component is a matter of great difficulty. (The production of 'pure' 'bottom' component on the other hand is simple.)

R. C. BACKUS: Another possible method of differentiating two particles of similar size but of different masses is suggested by an observation made some time ago by Robley Williams on influenza virus. On comparing electron micrographs of a field prior to shadow-casting with the same field after shadow-casting it was found that a number of particles appeared only on the shadowed one. This anomaly could best be explained on the assumption that the particles which did not appear on the unshadowed specimens were less dense than those that were visible.

R. MARKHAM: We have, of course, attempted experiments of this type some years ago, but without much success, probably because the size of the particles is rather small compared with that of the influenza virus. We intend to investigate the matter again, now that our microscope is equipped for high contrast observation. It is of interest to note that the shadow lengths of the virus particles are identical with those of the protein component showing that the latter has no tendency to collapse on drying.

AN ANALYSIS OF BACTERIOPHAGE MULTIPLICATION[1]

S. E. LURIA

Department of Bacteriology, University of Illinois

THE PROBLEM OF BACTERIOPHAGE MULTIPLICATION

The multiplication of bacteriophage, as well as that of all viruses, takes place inside the host cell. The new bacteriophage is not visible by electron microscopy on the bacterial surface nor is it accessible to antiphage serum action. The initial virus itself is sheltered from the neutralizing action of specific antiserum within seconds after its adsorption. Direct observation of phage-infected bacteria by either dark-field or electron microscopy yields results of little value. Indirect methods must be employed to analyze phage multiplication, and the interpretation of the data obtained by such indirect procedures is not only handicapped by factual uncertainties, but also beset with conceptual difficulties and semantic bias.

For example, such terms as virus 'synthesis' or virus 'reproduction' are often employed without qualification and, although perfectly legitimate in specific instances, are allowed to suggest unjustified and spurious analogies with the enzymatic synthesis of known metabolites, for instance, or with the reproduction of cells. It may be of value to state in operational terms some of the problems that face us in the study of virus multiplication, using as a model the multiplication of a phage accompanied by bacterial lysis, which will be the main subject of this paper.

Phage, like all viruses, consists of materials that differ chemically and genetically from that of the host cells. The chemical differences are not yet expressible in specific chemical terms, since they occur at the level of protein and nucleoprotein structure; but they can be traced operationally by the refined tool of immuno-chemistry. Specific antisera can discriminate between virus materials and host materials that cannot be separated from one another by the crude fractionation methods of present-day protein chemistry.

---

[1] Most of the original work by the author and his co-workers has been supported by grants from the American Cancer Society (upon recommendation of the Committee on Growth, National Research Council, U.S.A.) and from the University Research Board of the University of Illinois. Much credit for the current research described in this paper goes to Dr G. Bertani, Mr R. I. DeMars, Mrs M. L. Human, Dr F. Lanni, Dr Y. Théry Lanni, Miss S. J. Nice, and Miss M. R. Sheek, and to Dr C. Levinthal of the University of Michigan, who has collaborated with us in recent months. Many of the ideas presented here have originated or become crystallized in discussions with these workers, with other members of the author's laboratory and with bacteriophage workers in other laboratories, although the responsibility for all statements rests with the author alone.

A phage particle combines with a bacterial cell and several minutes later several hundred particles emerge. The problem of the origin of the new particles is a genetic and chemical one. What is the relation of the initial particle to the final ones? Does the former appear as such among the latter? Or does it contribute materials to the composition of one or more of them? If so, are these materials specific?

How does the initial particle preside over the formation and specificity of the new ones? Does it cause a transformation of a ready-made specific precursor into virus, or does it guide the synthesis of virus protoplasm from non-specific building blocks? If so, are these blocks supplied by the bacterial cell, by the external medium, or by both? Are they synthesized and assembled by mechanisms provided by the bacterium, or by the virus, or by both? What types of mechanisms are operative—enzymatic, autocatalytic, pattern-like, or other, less familiar ones? And how is the necessary energy obtained?

At what point in the formation of virus does the chemical specificity of the virus appear? Does a virus particle 'grow', that is, does it take in unspecific materials and produce more virus protoplasm within its own borders, then split into two or more particles? Or are there intermediate levels of organization, in which the building blocks, still free in the cell, are made more and more virus-like through a series of stages of increasing structural complexity and specificity? And, if such intermediate specific elements exist, what is their relation to the final virus particles? Are they assembled into virus particles, to which they contribute individual localized specificities, or does each of them represent a germ that grows into a full particle?

These questions concern the functional organization of the virus-infected cell. Cell organization, particularly at the level of the synthesis of specific cellular elements—genes, chromosomes, mitochondria and other cytoplasmic organelles—remains a field where even speculation has hardly dared venture. A virus, once inside its host cell, behaves as a component of an integrated functional cell system; yet, it retains its genetic and biochemical individuality. It is indeed the unique feature of viruses that they are chemically, serologically and genetically labelled cell components, which can be introduced into a cell from the outside under controlled conditions and can be followed by their labels in the labyrinth of cell transformations. Viruses in a sense are to cell physiology what isotopic tracers are to biochemistry. Viruses and especially bacteriophages appear therefore as a choice material for the analysis of cellular integration and of reproduction at the subcellular level.

The *lytic relation* between phage and bacterium, which will be my main topic, is not the only possible relation nor the most common. The *lysogenic relation,* characterized by the persistence of symbiotic phage-bacterium association over many cell generations, will be discussed by other participants in this Symposium. Even among the features of the lytic interaction, there are many important aspects, such as the process of phage adsorption by bacteria, that I shall not discuss insofar as they are not directly relevant to our theme, that of the mechanisms of virus multiplication. I shall confine myself to the events that intervene between the engulfment of a phage into its host, defined operationally by its becoming refractory to the neutralizing effect of phage antibodies in the medium, and the appearance of new phage in vastly increased amount. I shall discuss, together with the essentials of previous knowledge, some recent contributions to the kinetics, serology, and morphology of phage production.

THE RELATION OF PHAGE MULTIPLICATION TO THE ECLIPSE PERIOD

The most striking event following phage adsorption is the 'eclipse' of the phage as an infectious unit. No phage activity, defined as a material that can initiate phage production in new bacterial cells, can be extracted from the infected bacteria until around the middle of the latent period between infection and lysis (Doermann, 1951). This is true no matter what method is used to induce premature lysis of the infected bacteria. Later, phage activity begins to appear, at first as one or a few infectious units in some bacteria, then in amounts that increase at an approximately linear rate; the full yield is only reached at the time of lysis. The first active phage particles that can be extracted from bacteria after the eclipse are not the infecting ones, revived or reactivated after a period of dormancy. This extremely important conclusion rests mainly on the results of genetic analysis on the coli-phages of the T-even group.

In mixed infection with related phages differing by two or more hereditary characters there occurs a recombination of the characters of the infecting particles among the progeny according to precise genetic rules. The types of recombinants and their frequencies are characteristic for each given cross or mixed infection and can be interpreted, at least formally, in terms of a system of mutable determinants arranged linearly in at least three 'chromosomes' or linkage groups (Hershey & Rotman, 1949). In studying the frequency of genetic recombinants among the phage extracted by premature lysis from bacteria after mixed infection, Doermann (1951) found that the very first active phage extractable around the middle of the latent period already contained an appreciable proportion of recombinant particles and that this proportion was not much lower than that present in the final yield of phage upon natural lysis. This result indicates either that reproduction and recombination occur during the eclipse and that the reappearing phage activity consists of new phage particles, or that a large amount of recombination occurs before reproduction. The latter alternative would mean that the initial phage particles undergo recombination, then reappear and proceed to multiply. Such a sequence of events is made unlikely by the following line of evidence.

Spontaneous phage mutations occur during intracellular multiplication. A bacterium in which a mutation occurs liberates a mixture of normal and mutant phage particles. The number of mutant particles in the yield from such a bacterium must depend on the mode of phage multiplication, at least on the mode of multiplication of the portion of phage genetic material responsible for the mutant character in question. If a mutation has a constant probability of occurring every time a new replica is produced, the number of mutants to which each mutation gives rise in the final yield, and therefore also the frequency-distribution of mutants in individual bacteria, will depend on the sequence of replication. We have recently completed an analysis of the frequency distribution of all mutants T2*r* (large plaque type) and T2*w* (medium size plaque) in individual bacteria infected with phage T2 (Luria, 1951). The mutants occur in clones and the distribution of clone sizes is very close to the one expected from the assumption that reproduction occurs by a process formally analogous to binary fission. No mechanism assuming direct intervention of the initial phage in the production of each of the new phages fits the experimental results. These results represent substantial evidence for an exponential process of multiplication of genetic phage material, that is, for a process in which newly-formed individuals act in turn as models for replication.

Let us now return to genetic recombination. If instead of the distribution of spontaneous phage mutants we examine the distribution of recombinants in the yield of individual mixed-infected bacteria, we find that the recombinants are distributed more nearly at random than mutants would be (Hershey & Rotman, 1949; Doermann & Dissosway, 1949). The distribution found experimentally is incompatible with the hypothesis that an important amount of recombination occurs before reproduction. Rather, it seems to agree with the hypothesis that recombination occurs mostly in the later stages of replication.

Thus, since recombinants are present in relatively high frequency among the first active particles that reappear after the eclipse and yet seem to be formed mainly towards the end of phage reproduction, we conclude that the first active phage particles that reappear are themselves late products of reproduction. This amounts to suggesting that some reproduction of the genetic material of phage occurs during the eclipse period. It is interesting to recall that the same conclusion has been reached for phage T7 from an analysis of the evolution of radiation sensitivity of intracellular phage, that is, by a method completely independent of genetic analysis (Benzer, 1952).

GENETIC RECOMBINATION AND MULTIPLICITY REACTIVATION

We may stop here a moment to clarify one point. The results on the frequency distribution of spontaneous mutants concern individual phage properties. They tell us that individual genetic determinants reproduce exponentially; whether or not they do so all together, integrated into complete particles, we do not know. A few years ago I proposed a theory of phage multiplication according to which each genetic determinant or group of determinants reproduced independently and severally, the new determinants being then assembled into mature phage particles. This theory was proposed for the specific purpose of accounting quantitatively for a peculiar phenomenon of reactivation observed when a bacterium is infected with several particles of certain phages inactivated by ultraviolet light (Luria, 1947). It was postulated that inactivation was due exclusively to damage localized in discrete, genetically individualized elements of the phage, and that reactivation was due to the fact that genetic recombination could bring together full sets of undamaged genetic determinants to form fully active particles.

In order to explain quantitatively on this basis the very high frequency of reactivation, it was necessary to devise an extraordinarily effective means of recombination; I suggested therefore that each genetic element or unit reproduced independently and that the radiation-damaged units did not reproduce at all. Such a mechanism could lead to production of active phage whenever a full set of undamaged units was provided by the infecting particles as a group. Recent experiments (Dulbecco, 1952) have revealed new features of reactivation in multiple-infected bacteria, which cannot be explained in terms of the theory of genetic damage and repair by recombination. Especially, it appears that most reactivation results from some sort of co-operation among many damaged phage particles in carrying out some of the early stages of intracellular development. If genetic recombination is not the mechanism responsible for reactivation, the main reason for postulating an independent reproduction of individual genetic subunits disappears.

I think it is important to emphasize this point since the idea of independent reproduction and subsequent reassembly of genetic subunits, proposed as a working hypothesis for phage, has been seized upon eagerly by workers in other fields of virology and considered as 'proved' for phage. At present, phage experiments neither support nor exclude this working hypothesis; they simply do not require it. It is particularly important to realize this clearly when we state the hypothesis that phage reproduction takes place during a period in which phage is not recognizable as active particles. Reorganization resulting in eclipse of infectious particles and in reproduction of virus material in a non-infectious state is not synonymous with independent reproduction of genetic units.

CYTOCHEMISTRY OF PHAGE PRODUCTION

If reproduction can take place while infectious phage is not present, we must use methods other than activity measurements to reconstruct the reproductive process. One approach is a straight chemical one; although chemistry is as yet unable to distinguish phage protein or phage DNA from host protein or host DNA, it can trace the origin of the building blocks used in the synthesis of materials that appear in phage and can tell us about the role of various cell mechanisms in this synthesis. The results of the beautiful chemical work on *E. coli* B infected with the T phages (see review by Cohen, 1950 and 1951) can be summarized as follows:

The energy for phage synthesis is provided by the metabolic equipment of the host cell as it existed at the moment of infection; the same is true of most non-specific building blocks such as amino acids and nucleic acid components. If a bacterium requires tryptophan for growth, phage production in that bacterium will fail unless the amino acid is supplied. Part of the building blocks is synthesized anew after infection, part is provided by materials already present in the host cell at the moment of infection or brought in by infecting phage. The relative proportions of a phage constituent that are assimilated before and after infection depend on the phage and on the nature of the constituent. For example, of the DNA phosphorus of phages T2 and T6, about 25% comes from materials pre-existing in the host, 75% is assimilated after infection, while of the protein nitrogen about 90% is assimilated after infection. In the synthesis of phage T7 about 80% of the phosphorus comes from the host (Labaw, 1951).

Even more important, most materials that are transferred to the new phage from the host *or even from the infecting phage* appear to be first reduced to a non-specific level. Thus, about 30% of the phosphorus of the infecting phage appears in the phage progeny, and such a partial incorporation is repeated at successive transfers (Maaløe & Watson, 1951). This indicates that the parental contribution does not consist of the passage from generation to generation of a chemically stable genetic core.

As far as the bacterial contributions are concerned, there is evidence that the bacterial DNA is the major cell contributor to the phage building blocks; yet, the phosphorus and the various aromatic bases from host DNA are transmitted in different proportions to the phage DNA, which suggests that these materials are broken down at or below the nucleotide level before being utilized for phage synthesis (Kozloff, Putnam & Evans, 1950). It seems indeed likely that all contributions from various sources of building blocks suitable for phage synthesis become pooled together and that the mechanisms that assemble these non-specific building blocks into specific phage pieces utilize indiscriminately what is available at the moment. The bacterial contributions are actually exhausted fairly early; the proportion of pyrimidines derived from the medium after infection is lower in T6 phage particles formed early than in those formed very late (see Cohen, 1951).

All this concerns the origin and synthesis of the non-specific building blocks for the phage; it tells us little about the mechanism of formation of phage specific materials. In this formation the infecting phage must play a role, the nature of which still escapes us. Yet, in some cases at least, the phage producing mechanism is predominant in cells that are destined to lysis. The phage-coli cell complex makes protein, and, after a certain delay, DNA. These are virus components; RNA, which is an exclusive bacterial component, fails to increase. The infected cell keeps and uses pre-existent cell enzymes but apparently makes no new ones (Monod & Wollman, 1947).

This shift in the pattern of syntheses, beyond the level of the non-specific building blocks, to the formation of virus components with exclusion of several host components is a remarkable feature of the virus-cell complex. Its mechanism is obscure, although suggestive evidence has been afforded to indicate that the phage may play a role even at the enzymatic level. Thus, infection of *E. coli* B with phages of the T2 group induces a shift in the balance between types of carbohydrate metabolism from one favouring ribose formation to one that may favour desoxyribose synthesis (Cohen, 1951). At the level of complex syntheses, it is significant to find that infection with these phages can restore synthesis of DNA (and suppress synthesis of RNA) in bacteria that had lost the ability to form DNA as a result of treatment with nitrogen mustard (Herriott, 1951*a*). This suggests a direct, although unspecified role of phage in the synthesis of its nucleic acid from the non-specific blocks.

Some years ago we put forward the hypothesis that the shift in specific syntheses produced by phage infection may result from a replacement of the directive function of the bacterial nucleus with the directive function of phage itself (Luria & Human, 1950). Indeed, an almost immediate result of infection of *E. coli* B with a T phage is a disruption of the cytologically recognizable nuclear apparatus of the bacterium, followed later by the appearance of a granular chromatin, presumably representing the new phage. According to this view, the genetic material of a bacterium, collected in its nuclei, would normally be responsible for controlling the synthesis of specific cell materials (enzymes, antigens) from non-specific building blocks. The phage would eliminate the nuclear apparatus of the host and substitute for it its own, with a resulting shift in the specificity of the materials assembled thereafter.

Recent work on other organisms (*B. megatherium*, Lwoff *et al.*, 1950; Siminovitch & Rapkine, 1951; *P. pyocyanea*, Jacob, 1951) has shown in these organisms a situation different from that in *E. coli* B. Enzyme formation, enzymatic adaptation, RNA synthesis and increase in total cell material continue up to the time of lysis, both in sensitive bacteria infected from the outside and in lysogenic bacteria 'induced' by irradiation to release mature phage. The common feature in these cases and in *E. coli* B is an interruption of DNA increase upon infection or induction, followed by a new rise in DNA at a rate faster than the increase in total cell material. It seems likely that the alterations of DNA metabolism are the critical transformations connected with production of mature phage; their relation to nuclear disruption is unclear. The other effects on cell metabolism observed in *E. coli* B may reflect some peculiarity of this host, of the phages that attack it, or of both. It is possible that the suppression of cell growth and of enzyme formation upon phage infection is characteristic of phage-host combinations incapable of establishing lysogenicity. Comparative information on the cytochemistry and enzymatic activities of a variety of phage-host combinations seems greatly desirable.

SEROLOGICAL AND MORPHOLOGICAL ANALYSIS OF PHAGE PRODUCTION

We have seen that chemical studies can tell us about the nature and derivation of non-specific phage building blocks, but can reveal little about the existence and nature of intermediate stages between the building block and the phage particle. We have turned lately to the more refined tool of serology in an attempt to reveal materials with phage specificity before phage activity appears. This approach, of course, is similar to the one followed by a number of authors in the study of animal viruses, where the appearance of 'soluble antigens' and of other virus-specific elements has been studied in attempts to reconstruct the process of virus formation.

In our work we have used mainly a serum inhibition test, that is, a test for materials that can block the phage-neutralizing power of serum. In bacteria infected with some phages, for example T2 or T6, it is possible to demonstrate the presence of some phage-specific material during the eclipse period. This material consists of elements smaller than phage particles, since they pass through ultrafilters of 50 mμ A.P.D., while all phage activity is retained by ultrafilters of 75 mμ A.P.D. The ultrafiltrable material gives the same serological cross-reactions with heterologous phages as the active phage itself. It begins to increase in amount at the time of appearance of active phage or a little earlier, and it remains present side by side with active phage. Upon lysis, appreciable amounts of this ultrafiltrable material are liberated along with the phage and are found in the lysates, a fact that had been observed many years ago (Burnet, 1933), and that suggested to us the use of the serum blocking test in our work.

Complement-fixation tests give excellent and highly specific results with all phages that we have tested, particularly with purified materials. The ultrafiltrable material mentioned above has given no complement fixation in amounts that can block as much serum as about 10^9 phage particles, an amount of phage that fixes several units of complement.

Experiments on complement-fixing materials during bacteriophage infection require careful attention to discriminate between phage and bacterial antibodies, which are both present in sera prepared against partially purified phage. A recent report by Rountree (1951) of complement-fixation experiments on bacteria infected with phage T5 suggests a picture analogous to our findings with ultrafiltrate material, but claims that complement-fixing antigens completely disappear during the eclipse period. In Rountree's work, however, the infected bacteria were 'opened' by cyanide treatment, which fails to cause lysis if applied during the eclipse period (Doermann, 1951). Preliminary experiments in our laboratory using properly disrupted T2-infected cells (Y. T. Lanni, unpublished) reveal the presence of complement-fixing antigens throughout the eclipse period and their rise in amount in close parallelism to the appearance and rise of the mature phage.

These results and those with ultrafiltrable components indicate that phage antigens, non-identical and at least in part smaller than phage, are present during the eclipse phase and later. These observations parallel those made with other viruses (Hoyle, 1948; Henle & Henle, 1949). It is my opinion that in all cases it is premature and methodologically unsound to postulate that these antigens are virus precursors. For all we know, they might be products of abortive virus synthesis or breakdown products of surplus virus or of the real precursors.

Even less justified seem to be attempts to describe these virus antigens as *the* reproductive forms in cases of animal viruses, on the sheer basis of their presence and changes in amount. Only studies on the size, composition, mode of formation and disappearance of such virus-specific materials, integrated with many other approaches to the process of viral synthesis, will make it possible to determine the nature of these materials and their relation to virus multiplication. We are at present attempting to follow such a course in the case of bacteriophage and have recently succeeded by a lucky series of events in identifying one form of virus-related material, whose relation to the phage particle may be easier to determine with some certainty.

Several years ago Foster (1948) showed in our laboratory that proflavine, an acridine dye, used in sub-bacteriostatic concentrations prevents the production of active phages T2, T4, T5 and T6 without preventing lysis of the infected bacteria. In the presence of proflavine the cells lyse at the normal time but liberate no phage activity. In examining proflavine lysates for the presence of phage specific materials DeMars recently observed, besides the ultrafiltrable material (which is present in about the same amounts as in active lysates), a non-ultrafiltrable component, sedimentable in one hour at 20,000 *g* and endowed with little inhibitory power for phage-neutralizing antibody. Concentrates of this component were studied in the electron microscope by Dr C. Levinthal of the University of Michigan and revealed a new type of particle, round, collapsable and similar in size to the head of the particles of the corresponding phage (see Fig. 1).

Proflavine lysates often contain, besides a small number of tailed particles, almost as many tailless elements as there would be tailed particles in control lysates. The tailless particles are not adsorbed by phage sensitive bacteria. Although they have not yet been obtained completely free of admixed tailed particles, they are known to give precipitation and complement fixation with antiphage serum.

Electron micrographic counts of these particles were compared with the optical density of their suspensions for ultraviolet light and the results showed that the ultraviolet absorption coefficient per particle at 260 mμ is not more than one-tenth that of mature phage particles. This indicates

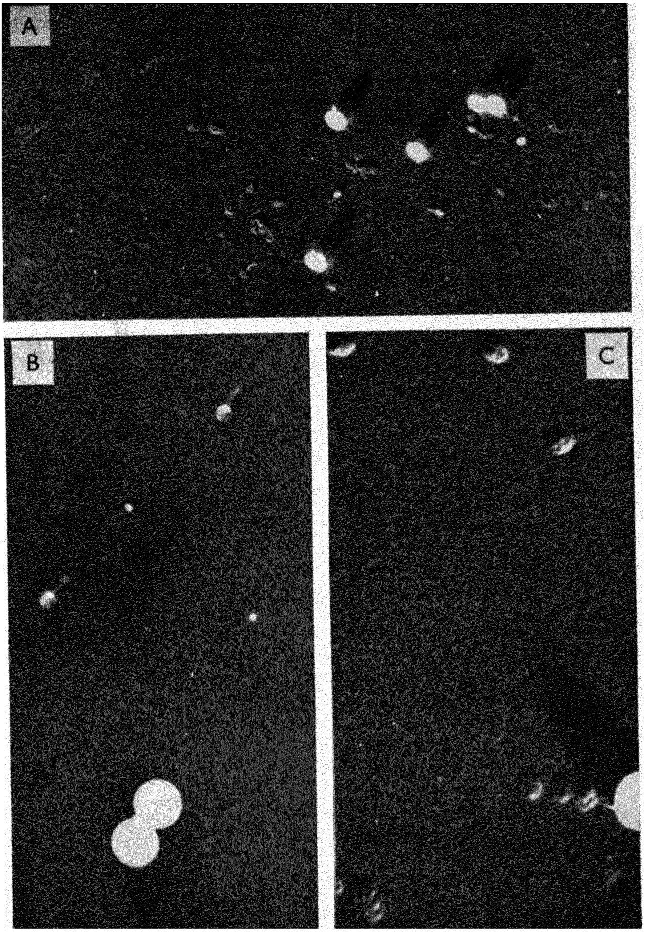

Fig. 1. A. Phage T2. A mixture of tailed phage particles and tailless particles produced in presence of 3·5 micrograms per ml. of proflavine. Note the difference in length between the shadows of the two types of particles. The large round particles are of polystyrene latex. × 14,000.

B. T2 phage particles. × 25,000.

C. T2 tailless particles. × 31,000.

that their nucleic acid content, if any, is much lower than that of mature phage particles.

There is evidence that these round tailless particles with low NA content are not artefacts and are indeed closely related to the mature phage. Electron microscopy on mechanically disrupted bacteria in the absence of proflavine, which has been carried out by Levinthal independently of our proflavine experiments, revealed a telling story. Using the decompression method of cell disruption (Frazer, 1951), which neither destroys nor inactivates active phage, Levinthal finds that bacteria disrupted during most of the eclipse period liberate no recognizable formed elements different from those found in non-infected bacteria; none, indeed, larger than 30 mμ. Then, towards the end of the eclipse period, there appear some round, tailless particles, identical with the proflavine particles described above, which in the next few minutes increase in number and are rapidly replaced by the regular, tailed, angularly-shaped phage particles. At the time of lysis only the latter particles remain visible.

These microscopic results, suggesting that the round particles are immediate precursors of the new phage particles, are in full agreement with what is known of the kinetics of proflavine action. The dye only blocks a late reaction in phage formation. Its effect can be completely suppressed by removing it about two minutes before the bacteria are due to lyse. Conversely, the dye is fully effective if added just before the time at which intracellular active phage begins to appear. If added later, the dye apparently limits phage production to the level of the active phage that was present at the time of its addition (Foster, 1948).

The finding of a particle resembling a tailless phage head without infectivity, and the evidence suggesting that this inactive particle is an immediate precursor of the active particle, agree with the conclusion reached from genetic experiments, which indicate that the replication of genetically specific phage material involves elements morphologically not recognizable as phage. According to this view, at the end of the eclipse period individual elements of this type would proceed to maturation through a stage-wise process, which includes the stage of round, tailless, particle and culminates into the formation of the mature, tailed particle. Once maturation has started, there would be present in a bacterium two populations, one consisting of immature phages and increasing by reproduction, the other consisting of mature phages and increasing by maturation of individual immature elements. Incidentally, the rate of the maturation process would not be informative as to the rate of immature phage reproduction.

The reproductive element might be identical with the symbiotic form of the phage in lysogenic bacteria, the 'prophage' of Lwoff's terminology (Lwoff & Guttman, 1950). The formation of active phage particles could then be considered as a maturation of individual prophages, which removes them one by one from participation in the complex series of reactions where synthesis, reproduction and genetic recombinations take place. Such an identity between the non-infectious reproductive forms of phage in lysogenic and in lysed bacteria, however, is not based for the time being on any factual evidence.

The maturation of individual particles may be visualized as the addition in each phage of some sort of 'cytoplasm', while the reproductive form would act as a nucleus. One might either consider the round, DNA-poor particles as already containing the nucleus, to which a DNA-rich cytoplasm would then be added; or one might consider the round particles as structural elements formed independently and to which at the very end of maturation the DNA-rich nuclei become attached (or at least become so firmly anchored as to be unremovable from them upon breakage of the cell).

Recent experiments by Hershey and Chase (1952), which became available after this paper was in press, partly clarify this picture. It had been discovered (Anderson, 1949; Herriott, 1951*b*) that osmotic shock separates from phages like T2 a phosphorus-free 'ghost', consisting of a flattened, apparently empty head skin and a presumably normal tail. The ghosts are still adsorbed by bacteria. Hershey and Chase, using isotopically labelled phage, showed that the ghosts contain practically all the phage sulphur and that upon attachment of a phage particle to a bacterium the ghost becomes separated from the phosphorus-containing portion of the phage. The latter apparently penetrates the bacterium, while the ghosts remain unchanged on the surface and take no part in reproduction; they can be removed from the surface of bacteria by violent mechanical agitation without interrupting phage reproduction. They contain the major antigenic components reactive with antiserum against whole phage particles.

The relation between the phosphorus-free skins or ghosts and the phosphorus-poor (or free) tailless immature elements is not yet clear. They differ, not only in morphology, but also somewhat in serological reactivity with phage antiserum. The round particles might be precursors of the phage skins or they might represent another phage component. The present estimates of the masses of the ghosts and of the tailless particles are not accurate enough to exclude their coexistence in a phage particle. Experiments in progress on tailless particles produced in media elled with S^{35} will reveal whether these elements contain all the phage phur as ghosts do.

hus, the phage cytoplasm appears to include, in the skin, the organ of ge attachment to the bacteria. It may also be involved in other ses of phage-host interaction that the reproductive phage elements not carry out alone. There is a suggestion of this in a comparison ween the relative frequency of lysis and maturation among bacteria t have just been infected with a phage and among those that become genic as a result of this infection. In the case of *Shigella dysenteriae* l phage P2 (Bertani, 1951), about 80–90% of the newly infected cells and liberate active phage, the other 10–20% become lysogenic. In progeny of the latter, maturation occurs in less than 1 out of 10,000 s per generation. The suggestion is that the cytoplasm of the infecting ticle may start, in over half the cells, the series of reactions that lead hage maturation and lysis. If it fails to do so, reproduction is limited he prophage stage, no new cytoplasm is formed and no maturation urs if not by rare accident.

PHENOTYPIC ALTERATION OF THE REPRODUCTIVE ABILITY OF PHAGE

ving summarized the evidence on the process of phage formation I h to conclude with a brief description of a remarkable case of pheno-ic, non-hereditary phage variation that is relevant to the preceding ussion (Luria & Human, 1952).

Vhen phages T2 or T6 infect bacteria of a certain group of mutants of *oli* strain B, they give rise to a yield of abnormal phage particles T*. se T* particles produce normal T2 or T6 on strains of *S. dysenteriae* fail to produce any active phage if adsorbed by cell of *E. coli* B or of of its mutants. The coli cells are killed but fail to lyse. The variation n the normal to the T* form is strictly phenotypic and transitory; one wth cycle of T* in *S. dysenteriae* yields perfectly normal phage T2 T6), fully active on *E. coli* B.

n interesting aspect of this phenomenon is its relation to bacterial siology. The failure of T* to reproduce in *E. coli* cells is only com-e if the latter are in the exponential growth phase; if the bacteria are ing old cells infected in non-nutrient medium, about 5 to 10% of the cting phage succeeds in reproducing. Incubation of starved cells with led nutrients before infection, rapidly suppresses their ability to support wth of T*.

he importance of these observations for virus multiplication is two-l. First, the dependence of T* reproduction on the physiology of the coli host suggests an inhibition on phage growth on the part of the synthetic machinery of the host, an inhibition that is less complete in starved, non-synthesizing cells. Second, the formation of T* instead of normal phage in the mutant host shows a host-induced transformation of the phenotype of a virus. Such a control by the host genotype over the phenotypic expression of a genetic potentiality of a virus is a novel situation in virology. Formally at least, this finding supports the suggestion that a phage consists of a genetic component or 'nucleus', which determines its genetic potentialities, and of a physiologically active portion or 'cytoplasm', through which these potentialities become manifested in various phases of virus-host interaction.

REFERENCES

Anderson, T. F. (1949). The reactions of bacterial viruses with their host cells. *Bot. Rev.*, **14**, 464.

Benzer, S. (1952). Resistance to ultraviolet light as an index to the reproduction of bacteriophage. *J. Bact.*, **63**, 59.

Bertani, G. (1951). (To be published.)

Burnet, F. M. (1933). A specific soluble substance from bacteriophages. *Brit. J. Exp. Path.*, **14**, 100.

Cohen, S. S. (1950). Growth requirements of bacterial viruses. *Bact. Rev.*, **13**, 1.

Cohen, S. S. (1951). The synthesis of nucleic acid by virus-infected bacteria. *Bact. Rev.*, **15**, 131.

Doermann, A. H. (1951). Intracellular phage growth as studied by premature lysis. *Feder. Proc.*, **10**, 591.

Doermann, A. H. & Dissosway, C. F.-R. (1949). Intracellular growth and genetics of bacteriophage. *Carnegie Inst. Wash. Yearbook*, **48**, 170.

Dulbecco, R. (1952). A critical test of the recombination theory of multiplicity reactivation. *J. Bact.*, **63**, 199.

Foster, R. A. C. (1948). An analysis of the action of proflavine on bacteriophage growth. *J. Bact.*, **56**, 795.

Frazer, D. (1951). Bursting bacteria by release of gas pressure. *Nature*, **167**, 33.

Henle, W. & Henle, G. (1949). Studies on host-virus interactions in the chick embryo-influenza virus system. III. Development of infectivity, haemagglutination, and complement fixation activities during the first infections cycle. *J. Exp. Med.*, **90**, 1.

Herriott, R. M. (1951*a*). Nucleic acid synthesis in mustard gas-treated *E. coli* B. *J. Gen. Physiol.*, **34**, 761.

Herriott, R. M. (1951*b*). Nucleic-acid-free T2 virus 'ghosts' with specific biological action. *J. Bact.*, **61**, 752.

Hershey, A. D. & Chase, M. (1952). Independent functions of viral protein and nucleic acid in growth of bacteriophage. *J. Gen. Physiol.* (in the press).

Hershey, A. D. & Rotman, R. (1949). Genetic recombination between host-range and plaque-type mutants of bacteriophage in single bacterial cells. *Genetics*, **34**, 44.

Hoyle, L. (1948). The growth cycle of influenza virus A. A study of the relations between virus, soluble antigen and host cell in fertile eggs inoculated with influenza virus. *Brit. J. Exp. Path.*, **29**, 390.

JACOB, F. (1951). Adaptation enzymatique pendant le développement du bactériophage chez *Pseudomonas pyocyanea*. *C. R. Acad. Sci.*, **232,** 1780.

KOZLOFF, L., PUTNAM, F. W. & EVANS, E. A., Jr. (1950). Precursors of bacteriophage nitrogen and carbon. California Institute of Technology, *Viruses*, 1950, ed. M. Delbrück, p. 55.

LABAW, L. W. (1951). The origin of phosphorus in *Escherichia coli* bacteriophages. *J. Bact.*, **62,** 169.

LURIA, S. E. (1947). Reactivation of irradiated bacteriophage by transfer of self-reproducing units. *Proc. Nat. Acad. Sci., Wash.*, **33,** 253.

LURIA, S. E. (1951). The frequency distribution of spontaneous bacteriophage mutants as evidence for the exponential rate of phage reproduction. *Cold Spring Harbor Symp.*, **16** (in press).

LURIA, S. E. & HUMAN, M. L. (1950). Chromatin staining of bacteria during bacteriophage infection. *J. Bact.*, **59,** 551.

LURIA, S. E. & HUMAN, M. L. (1952). *J. Bact.* (in the press).

LWOFF, A. & GUTTMANN, A. (1950). Recherches sur un *Bacillus megatherium* lysogène. *Ann. Inst. Pasteur*, **78,** 711.

LWOFF, A., SIMINOVITCH, L. & KJELDGAARD, N. (1950). Induction de la production de bactériophages chez une bactérie lysogène. *Ann. Inst. Pasteur*, **79,** 815.

MAALØE, O. & WATSON, J. D. (1951). The transfer of radioactive phosphorus from parental to progeny phage. *Proc. Nat. Acad. Sci., Wash.*, **37,** 507.

MONOD, J. & WOLLMAN, E. (1947). L'inhibition de la croissance et de l'adaptation enzymatique chez les bactéries infectées par le bactériophage. *Ann. Inst. Pasteur*, **73,** 937.

ROUNTREE, P. M. (1951). A complement-fixing antigen of Bact. coli bacteriophage T5: its behaviour during virus growth. *Brit. J. Exp. Path.*, **32,** 341.

SIMINOVITCH, L. & RAPKINE, S. (1951). Modifications biochimiques au cours du développement des bactériophages chez une bactérie lysogène. *C. R. Acad. Sci.*, **232,** 1605.

DISCUSSION

J. D. WATSON: I should like to amplify the remarks of Dr Luria concerning the recent experiments by Hershey and Chase which have been communicated to me. Using phage isotopically labelled with S^{35} or P^{32} they have found that osmotic shock disrupts particles of phage T2 into material containing all the phage sulphur in a form precipitable by anti-phage serum, and capable of specific adsorption to bacteria. On the other hand, the phage DNA as measured by P^{32} is released into solution in a form not precipitable by antiserum and not adsorbable to bacteria. From these experiments they conclude that the sulphur-containing protein of the phage particle functions as a membrane that protects phage DNA from DNA-ase, comprises the sole or principal antigenic material and is responsible for attachment of the virus to the bacteria. They have further demonstrated that following infection of labelled phage, the sulphur-containing virus protein can be largely removed from infected cells by the shearing force of a Waring blendor while only a small fraction of the phage phosphorus is removed. Despite the removal of most of the sulphur-containing proteins, the infected bacteria remain capable of yielding phage progeny. Hershey and Chase therefore conclude that following infection, most of the phage sulphur remains at the cell surface while the phage DNA enters the cell. They further find that the phage progeny yielded by bacteria infected with S^{35} labelled phage contain less than 1% of the parental S^{35}. This is in sharp contrast to the results of experiments with P^{32} or C^{14}-adenine labelled phage (Watson & Maaløe, in the press) in which the progeny particles contain 40–50% of the parental phosphorus.

In their manuscript Hershey and Chase draw attention to the fact that their experiments refer only to the fraction of the virus protein which contains S^{35} and suggest that different results might be obtained if another isotope were used to detect the virus protein. To test this point Dr O. Maaløe and I last summer, at the State Serum Institute in Copenhagen, followed the fate of phage T4, labelled by growth in a medium containing carboxyl tagged C^{14}-glycine. In these experiments we used a mutant of *Escherichia coli* strain B which was unable to synthesize any purine bases, since previous biochemical work has shown that glycine is a precursor of the purine ring. We thus believe that our phage was labelled largely in its protein component, though it is possible that both the pyrimidine and the desoxyribose components were also labelled. Unfortunately, due to the low specific activity of the phage, we did not attempt to determine chemically the distribution of the C^{14}.

When this labelled phage was used to infect unlabelled bacteria, we found that over 50% of the infecting isotope remained attached to the bacterial membrane while only 10–15% appeared to be transferred to the progeny phage. We are not sure of the reliability of this apparent low transfer to the progeny since the low specific activity prevented us from highly purifying the progeny particles. Thus we can only conclude that the transfer is not higher and may be much lower if the progeny virus preparations were not pure. Our results thus show a close parallel to those of Hershey and Chase with S^{35} and strongly suggest that their S^{35} conclusions will apply to all of the virus protein. It is tempting to conclude that the virus protein functions largely as a protective coat for the DNA and that the perpetuation of genetic specificity is largely or entirely a function of the DNA.

At the same time as we did the experiments with C^{14}-glycine, Dr Maaløe and I did a number of experiments on the transfer of nucleic acid from parental to progeny phage. Our results can be summarized as follows:

1. Infection of unlabelled host bacteria by P^{32} labelled phage T4 results in the transfer of approximately 40–50% of the parental P^{32} to the progeny phage. Five to ten per cent of the original P^{32} is associated with bacterial debris while the remaining 40–50% is present in TCA-precipitable material too small to be sedimented by high speed centrifugation. The transmitted P^{32} is actually incorporated into the progeny particles and not merely attached to the virus surface. This transfer value of 40–50% is extremely reproducible, generally varying less than 5% from experiment to experiment. Virus preparations prepared at different times and in different laboratories yield identical transfer values, rendering unlikely the existence of a variable fraction of 'dead' particles which cannot transfer its P^{32}.

2. For P^{32} transfer to occur, the infecting particles must adsorb almost simultaneously to the same bacterium. Particles adsorbing to a bacterium to which a previous particle had adsorbed two or more minutes previously do not transfer any of their P^{32}. Instead only 50% of the P^{32} of these secondarily adsorbed particles remains attached to the host bacteria; the remaining 50% is broken down into TCA-soluble material and released within 2 or 3 minutes after infection into the surrounding medium. In contrast over 95% of the P^{32} of those particles which transfer P^{32} is retained within the infected bacteria until the time of lysis. Under conditions of simultaneous adsorption, the transfer value per infective particle is unchanged by varying the number of adsorbed particles per bacterium from one to ten.

3. The transfer value of P^{32} labelled phages T2, T3 and T6 is also between 40 and 50%. As T3 is unrelated to the T2, T4 and T6 group of related phages, it is possible that an incomplete P^{32} transfer of approximately one half is a general characteristic of phage reproduction.

4. The transfer of phage purines labelled with C^{14} is also 40–50%. This suggests that the purine bases and P are not transferred independently but as the constituents of mono-or poly-nucleotides.

5. The parental isotopes are not transferred in equal amounts to all of the progeny particles; instead, over 50% of the parental isotope is transmitted to the first 50 progeny particles which mature. An increase in burst size from 150 to 350 due to lysis inhibition does not significantly increase the transfer percentage. Thus the incomplete isotope transfer is not due to isotope transfer to immature progeny particles which fail to mature because of the onset of spontaneous lysis.

6. The isotope transfer from progeny particles labelled by infection of unloaded bacteria by labelled phage is 40–50%, that is identical to the transfer from phage uniformly labelled by growth in labelled bacteria (the second generation experiment). The progeny particles must therefore also be labelled uniformly.

7. Phage heavily damaged by X-rays or excluded from growth by the simultaneous adsorption of an unrelated phage can still transfer part of their nucleic acid. Isotope transfer is therefore not necessarily connected with the transfer of genetic specificity.

I believe two main conclusions should be drawn from these results: (1) While the transfer of nucleic acid is incomplete, it is always between 40 and 50%. This suggests that the nucleic acid of *each* infecting particle is divided into two parts of which only one is transferred. (2) The equivalence of nucleic acid transfer from parental and progeny particles merely indicates a similarity of the isotope distribution, and does not provide evidence as to the mechanism of transfer. A specific transfer of genetic specificity can be reconciled with our data by assuming that the genetic material is equally distributed in the transferred and non-transferred regions of the phage. Our experiments therefore fail to differentiate between a transfer in the form of large blocks of DNA (genes, chromosomes?) or after complete break-down and reassimilation.

Thus we cannot yet say whether a transfer of genetic specificity is accompanied by a transfer of intact parental genetic material.

D. KAY: You (J. D. Watson) have mentioned some work by Dr Hershey in which phage was labelled with S^{35}. During the multiplication of this phage the radioactivity was not transferred to the progeny. Do you consider this to be a fair test for the lack of transfer of the protein from the infecting virus to its progeny as sulphur-containing amino acids compose only a small fraction of the virus protein?

J. D. WATSON: Our meagre evidence on the composition of the phage protein(s) does not allow us to draw any final conclusions on protein transfer. The similarity of the results of Maaløe and myself with C^{14}-glycine to those of Hershey with S^{35} suggests that much of the virus protein behaves as the sulphur-containing fraction. We shall have to do experiments with additional labelled amino acids before we can answer your question.

L. F. HEWITT: Is it possible that in a phage particle the nucleic acid and protein portions are counterparts linked together so that the nucleotides in the nucleic acid molecule fit into complementary amino-acid side chains in the protein? Thus when the phage particle is specifically adsorbed by a susceptible bacterial cell the nucleic acid and protein components hinge apart, the protein portion anchoring on to a section of the bacterial surface of complementary structure while the nucleic acid is liberated and enters the cell. This might account for the protein 'ghosts' left on the bacterial surface during phage multiplication. The nucleic acid entering the cell may control the synthesis of specific protein in virtue of its counterpart structure, and perhaps also the newly synthesized protein influences the synthesis of complementary nucleic acid molecules. Thus the chain of synthesis would continue until the new protein and nucleic acid molecules begin to unite forming complete phage particles which appear only at the end of the latent or 'eclipse' period.

J. D. WATSON: As a result of Hershey's recent work, I think it more probable that the nucleic acid is not specifically attached to the protein component by salt-like linkages but that instead the relationship between these two components is like that of a hat inside a hat box. The evidence on this point is certainly far from conclusive (*vide J. D. Watson* on *A. Lwoff*).

N. W. PIRIE: Although some bacterial viruses indubitably have tails after being dried for electronmicrography, I remain sceptical about their tails when in the wet and natural state. It seems possible that they have a mucus coat that gets drawn out in this way on drying and I would like to ask whether it has been demonstrated that the sulphur of the skin and tail, which seems not to be absorbed by the bacterium when the latter is infected, is really protein sulphur. If it turned out to be ester sulphate it would be rather in favour of the alternative view.

J. D. WATSON: In Hershey's letter to me he does not state his experimental evidence for believing that the S^{35} label is specifically located in the protein component. I suspect he has demonstrated this point by chemical isolation of the sulphur-containing amino acids.

D. E. DOLBY: The ability of desoxyribonucleic acid to reverse proflavine inhibition of T2 synthesis is not in itself evidence that proflavine inhibits desoxyribonucleic acid synthesis, as Professor Luria implied, however likely this might be on other grounds. In view of the ease with which proflavine forms complexes with nucleic acids, it is apparent that any external desoxyribonucleic acid added to the system would combine with the proflavine to form a complex and thus remove it from the site of its inhibitory action on the enzyme, whether this is concerned with synthesis of desoxyribonucleic acid or not.

A further property of proflavine which does not appear so far to have been pointed out is that it causes photo-inactivation of phages, or at least of phage T2, in the isolated state but not when adsorbed on bacteria. This might help to explain the very large variation in the concentration of proflavine reported by various workers to be required for inactivation of different phages.

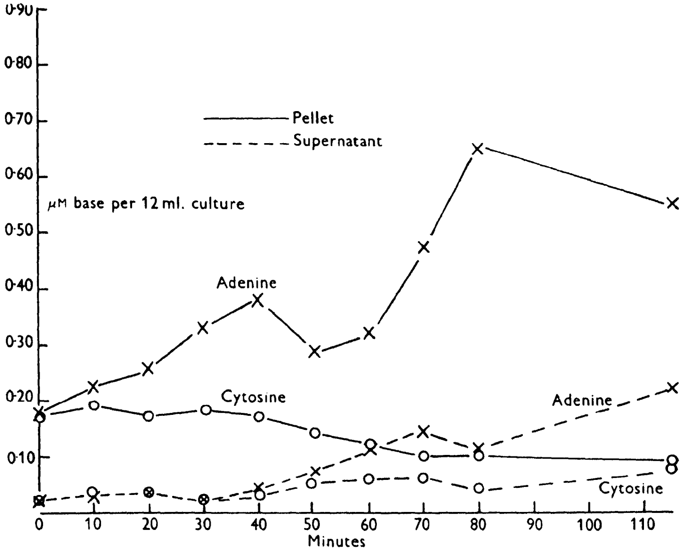

Fig. 2

J. E. HOTCHIN: Dr Luria has stated 'Phage, like all viruses, consists of materials that differ chemically and genetically from that of the host cells. The chemical differences are not yet expressible in specific chemical terms, since they occur at the level of protein and nucleoprotein structure; but they can be traced operationally by the refined tool of immunochemistry.'

It is possible to add to the paragraph quoted, some information which will indicate that chemical approaches to the problems of virus synthesis are already yielding information about the synthesis of virus material chemically distinguishable from similar host constituents.

I have been applying recent techniques of chemical analysis to the study of cells of *B. coli* infected with T2*r*+phage. The infected organisms were centrifuged down from their broth culture after addition of formaldehyde to a concentration of 2%, at 10 minute intervals after infection with phage. The RNA of the pellet of organisms was first removed by alkali and subsequently the DNA was extracted in acid. The DNA extract was then hydrolysed and the constituent bases measured quantitatively by paper chromatography and U.V. spectrophotometry. Applied to the purified T2, results showed the phage to contain little or no cytosine whereas in the *B. coli* DNA, cytosine and adenine contents were almost the same.

This fact was used to follow the increase in intracellular phage DNA during the latent period of infection. Fig. 2 shows the results of the experiment described above in terms of the adenine and cytosine content of the pellet of infected organisms. The dotted lines show corresponding figures for the supernatant broth.

Fig. 3 shows the result of plotting the adenine content after subtracting the cytosine equivalent of host DNA present in each extract.

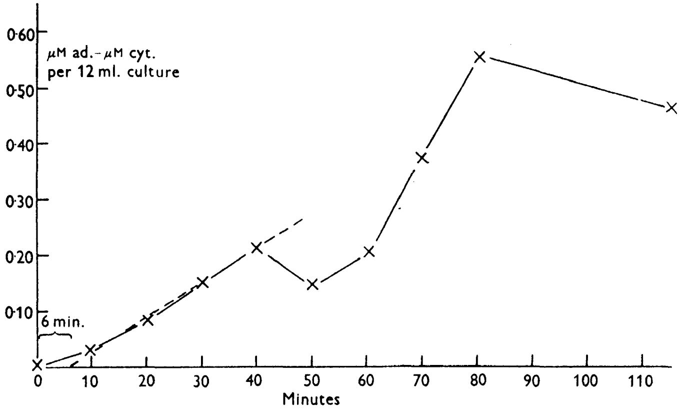

Fig. 3

This value gives the amount of adenine which is present in excess of that accountable as host DNA, and represents the adenine of the virus DNA, which should be proportional to the total synthesized virus DNA.

Too few experimental points are present for a precise analysis of the curve, but a straight line drawn through the existing points crosses the time scale at a point six minutes after infection.

The dip in the curve at 40 minutes may be due to the initial lysis of a proportion of the organisms, with subsequent continuation of DNA synthesis by the remainder, due to lysis inhibition. Further experiments are being made.

THE ROLE OF MUTATION IN THE SURVIVAL AND MULTIPLICATION OF BACTERIAL VIRUSES

J. S. K. BOYD

Wellcome Laboratories of Tropical Medicine, London

By general agreement many workers in the United States and elsewhere who are engaged in the study of bacterial viruses (phages) have confined their investigations to the 'coliphages', a group collected from different sources which are all active against *Escherichia coli* B. As a result of this co-ordinated action a store of information has been amassed about these particular phages which, in its basic principles, can be applied to other phages of similar types and also to certain plant and animal viruses. Few subjects can have been more thoroughly or more carefully investigated.

Unfortunately, concentration on this somewhat narrow front has tended to divert attention from other equally important aspects of the 'life history' of some, if not all, of these phages. The phenomenon of the lysogenic bacterium has long been recognized and at one time was widely studied, but since the investigations of Burnet and his colleagues (Burnet, 1932; Burnet & McKie, 1929; Burnet & Lush, 1936) little has been done to assess its significance, and until recently no serious attempts have been made to correlate the 'latent' or 'natural' or, as they will be designated in this paper, the 'symbiotic' phages which cause lysogenicity with the more spectacular 'lytic' types such as the coliphage group. Important contributions to this subject have now been made by Lwoff and others (Lwoff & Gutmann, 1950; Lwoff, Siminovitch & Kjeldgaard, 1950), who have investigated a lysogenic strain of *Bacillus megatherium*. These workers consider that in this strain the phage particle within the bacterium is arrested in its development at the stage which they term 'probacteriophage', and in this form divides and is passed on into each daughter bacterium. From time to time some stimulus causes certain changes to occur in the bacterium which allow the probacteriophage to resume its interrupted development and pass through the different stages which end in the destruction of the bacterium and liberation of free phage particles. According to this theory, symbiotic and lytic phage represent different levels in the development of one and the same type of phage particle, the progress from symbiotic to lytic being primarily determined by metabolic changes in the host bacterium, to which the response of the phage particle is secondary.

However this may be in the case of *B. megatherium*, investigations carried out with lysogenic *Salmonella typhi-murium* in the last four years suggest that the breakdown of the symbiotic balance between phage and bacterium leading to the onset of destructive phage multiplication is not primarily attributable to the bacterium but to a mutation which fundamentally changes the properties of the phage (Boyd, 1951*a*). Further, a reversion from the lytic mutant to the symbiotic prototype has also been observed in certain of these phages (Boyd, 1951*b*). This alternation of phases provides a mechanism which plays an important part in ensuring the survival of the phage.

This paper give some account of observations made on these broad and general issues, and outlines a complex life history which must be studied in its entirety if a full understanding of the mechanism of phage reproduction is to be reached.

PHAGES AND INDICATOR STRAINS

In the investigations to be described, a series of strains of the Type 'A' phages found in association with *S. typhi-murium* (Boyd, 1950) has been used. These were recovered either from samples of infected faeces or from pure cultures of *S. typhi-murium*. Most of the experiments were carried out with Type A1, for which the indicator strain, *S. typhi-murium* 1404, was used, but all observations were confirmed with Types A2 and A3. The methods employed have been fully described elsewhere (Boyd, 1950, 1951*a*).

FUNDAMENTAL CHARACTERS OF LYSOGENIC *S. TYPHI-MURIUM*

The 'lysogenic' culture—the strain of *S. typhi-murium* symbiotically infected with Type A phage—has characters of considerable interest and importance.

Morphologically the organisms show no abnormality. They are actively motile and divide normally. When examined by special staining methods or by phase-contrast illumination (Boyd, 1949) the internal structure of the bacillus presents the same appearances as that of an uninfected organism. There is no dispersal of the nucleus such as is found in organisms infected with lytic phage (Luria & Human, 1950). Turbidity curves of growing broth cultures show slightly more active growth than those of the uninfected organism. The biochemical and serological reactions reveal nothing unusual.

Every organism in such a culture is infected. Colonies which develop from the bacteria are normal in appearance and show neither 'nibbling' nor translucent patches. Yet every one of them is lysogenic. Although various methods have been tried, it has so far proved impossible to 'cure' bacteria infected with this phage.

Despite the outward normality of a culture, free phage particles can always be demonstrated in it. The mechanism by which these particles are liberated, previously obscure, has been elucidated by Lwoff & Gutmann (1950), who, by means of microculture, have shown, in the case of *B. megatherium*, that whilst the majority of bacteria grow and divide normally an occasional organism bursts, liberating phage particles. *S. typhi-murium* has not been examined by this very ingenious micro-culture technique, but results given by less precise methods are in general agreement and leave no doubt that the same mechanism is in operation.

All efforts to recover symbiotic phage from a lysogenic culture by dissolving or otherwise breaking up washed bacteria have been unsuccessful. In the writer's laboratory this has been attempted by the use of a lytic phage of a different type and, less efficiently, by mechanical grinding. It would appear that the symbiotic phage is so closely integrated with the bacterium that it cannot survive the latter's death. Further evidence of this assumption is afforded by the reaction to heat. In the free state, phage A will resist a temperature of 80° for 30 minutes. In the symbiotic state it is destroyed, in company with its host, when exposed for the same time to a temperature of 55°.

The organisms of a lysogenic culture can adsorb the lytic particles, either of the first or subsequent generations, to which the culture gives rise, but are resistant in that they are not lysed.

As every daughter cell in a multiplying lysogenic culture is infected with symbiotic phage, it follows that the phage must divide as fast as the bacterium. Discussion on the location of the phage in the bacterium and on whether one or more than one particle is present in each bacterium will be deferred to a later stage.

α LYSATE

The characteristic feature of a lysogenic culture is the liberation of particles of active phage. These are found in the supernatant fluid of a spun broth culture of an infected organism. For convenience of description, and to differentiate it from other lysates which will be described in due course, this will be termed α lysate. The plaques formed by phage particles in a lysate are studied by flooding an indicator plate (i.e. a plate of nutrient agar sown with a broth culture of the indicator organism and dried) with about 0·5 ml. of lysate diluted if necessary, pipetting off the excess, drying the plate and incubating overnight. Three varieties of plaque are produced by the action of α lysate. These are mature, solid-centre plaques: partially grown or immature versions of the same type: and relatively clear-centre lytic plaques (the reason for the designation 'lytic' will later become apparent).

Mature solid-centre plaques

The majority of the plaques formed by Type A phages are of typical appearance (Fig. 1). Surrounding a central disc of bacterial growth is a narrow, relatively clear rim, outside which is a halo of varying width. In vertical section, the central disc in the plaque appears as a flat dome set in a saucer-shaped depression. The plaque grows in a characteristic fashion which is related to other phenomena to be detailed later. It appears first as a small, clear spot. As it increases in size, a pin-point of bacterial growth is seen at its centre. Both clear area and central spot enlarge, so that the former becomes a ring round the latter. Finally, the halo develops. These characters become sharper when the plate is incubated until bacterial growth is arrested. (All the stages of plaque development are to be seen in Fig. 3). The size of the plaque is governed by the rapidity of the centrifugal spread of the phage in relation to the rate of growth of the indicator bacteria. Starting from a central point, the particles spread outwards in a series of waves corresponding to the bursts of infected bacteria. So long as they come into contact with actively multiplying bacteria, infection and a further cycle of development ending in lysis occurs in all but a minority of the organisms which are infected. The limit of this centrifugal spread is reached when the phage comes to the point where the multiplying organisms have piled on top of each other to such an extent that the upper layers are deprived of nourishment from the underlying medium and have stopped growing. As phage can develop to the point of lysis only in growing organisms, these latent bacteria remain intact and so form an edge to the plaque. The halo is produced by an undermining action of the phage which continues to spread centrifugally and produce lysis in the growing bacteria in contact with the medium, stopping only when bacterial growth reaches its limit and ceases. The increasing accumulation of inert and hence intact bacteria in the upper layers accounts for the shelving edges which produce the halo.

So far, this description might apply equally to the clear plaques produced by pure lytic phages, but the 'solid' centre has still to be explained. If a suspension of the bacteria of which it is formed be plated it will be found that they are lysogenic—i.e. symbiotically infected—and hence resistant to the action of the lytic particles. As the plaque took origin

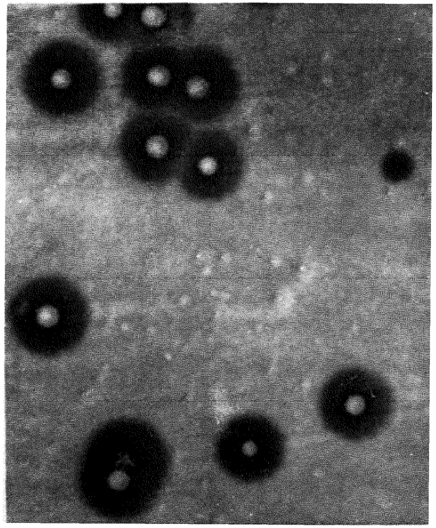

Fig. 1. Plaques on an indicator plate flooded with β lysate. The majority are of uniform size and appearance. This particular field has been selected to show both an immature plaque and a plaque developing from a lytic mutant. × 5.

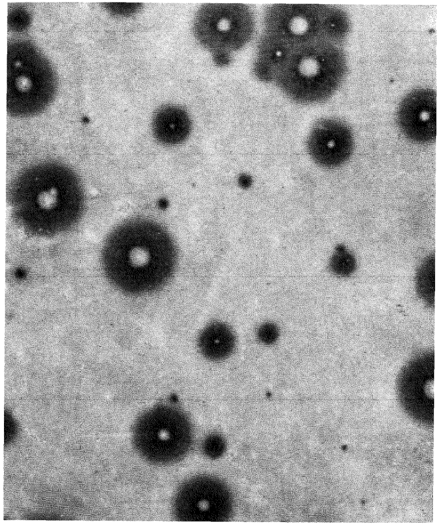

Fig. 2. Plaques on an indicator plate flooded with α lysate (heated to 58° for 30 min.). Plaques at different stages of development are seen. These all develop from the action of free phage particles. × 5.

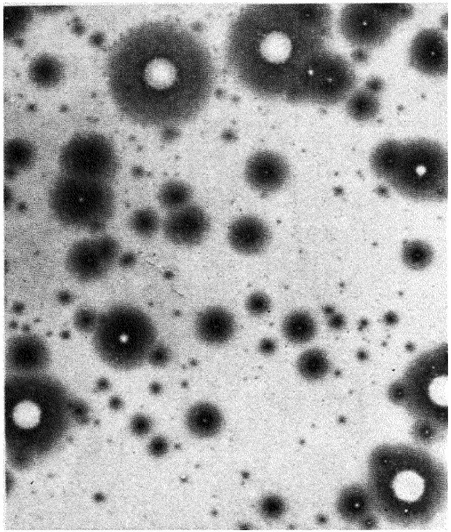

Fig. 3. Plaques on an indicator plate flooded with diluted α lysate (spun but not heated). Plaques have developed both from free phage particles and from lysogenic bacteria. ×5.

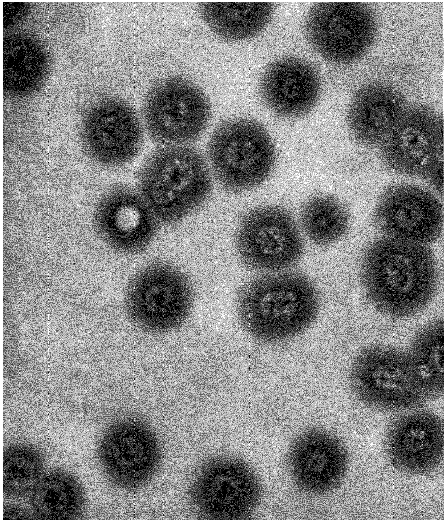

Fig. 4. Plaques on an indicator plate flooded with γ lysate. All are of the lytic type (clear centre stippled with granules) with the exception of one (very rare) β mutant showing the typical 'solid-centre'. ×5.

from a single particle with lytic properties, it follows that some of the offspring of this particle have produced symbiotic infection in certain indicator organisms. It is these which have subsequently multiplied to form the solid centre.

The sequence of events in the development of a solid-centre plaque is, therefore, primary lysis, followed by secondary growth of symbiotically infected, phage-resistant bacteria.

The virus particles which produce these solid-centre plaques will be called β particles.

Immature plaques

A characteristic feature of α lysate is the disproportionate number of immature plaques which it produces. These are of all sizes from minute clear spots up to almost fully grown plaques (Fig. 2). The relative proportions of mature and immature plaques vary in different strains. Cultures of the smallest of these plaques develop in the same way as cultures of the most mature. The particles are, therefore, qualitatively alike in both. The difference in plaque size is most probably related to the length of time the plaque has been growing. The significance of this observation will be discussed in due course.

Lytic plaques

The number of particles producing lytic plaques which are to be found in α lysate varies in different strains of lysogenic bacteria. In some they are relatively common, i.e. one or two in a plate containing about 1,000 solid-centre plaques. In others, there may be only one lytic plaque to 5,000, or even 10,000 of the solid-centre variety.

The appearance of the lytic plaque is characteristic (Figs. 1 and 4). It is approximately the same size as the solid-centre plaque and has a similar halo. The centre, instead of being covered with a dome of bacterial growth, shows a clear background on which are varying numbers of small granules or colonies, sometimes scanty, sometimes numerous. Plates made from a suspension of the organisms in the granules yield a mixed crop of colonies. Some are heavily phage eaten (i.e. sensitive and contaminated by extraneous phage while developing) while others are normal in appearance. Bacteria in the majority of these normal colonies are sensitive to the action of the phage. It would, therefore, seem that phage and bacteria have been growing together in some sort of 'external' balance. A similar phenomenon has recently been recorded by Weigle & Delbruck (1951). A minority of the normal-looking colonies are composed of bacteria which are permanently resistant to the phage. This resistance is *not* due to symbiotic infection but it is of the familiar type developed in response to the action of lytic phages. Lysogenic bacteria have never been recovered from the granules in lytic plaques.

These lytic plaques resemble in their principle characters the 'N' phages described by Burnet (1930), differing only in that Burnet found his 'N' phages did not produce resistant bacterial variants. As these are scanty—if not absent—in young cultures, it is possible they may have been overlooked.

The particles which produce these lytic plaques will be called γ particles.

β LYSATE

When a small quantity of α lysate is added to a culture of *S. typhimurium* growing in broth it will, after two to three hours' incubation, bring about complete or almost complete clearing. The resultant liquid, after being heated and, if necessary, spun, will be found to contain approximately 10^{10} β particles per ml. This will be referred to as β lysate. This produces the same three types of plaque as does α lysate but in different proportions (Fig. 1). Solid-centre plaques predominate and, unlike those in α lysate, are uniform in size. Otherwise they are identical with similar plaques formed by α lysate. Immature plaques occur, but are much more scanty than in α lysate, and rarely number more than about 5% of the mature plaques. Lytic plaques occur with about the same frequency as in α lysates. In some strains several plates had to be examined before one was found, but in none of the strains investigated (some twenty-eight in all) have we failed to secure a mutant of this type.

γ LYSATE

γ lysate is produced in the first instance by making a culture from a lytic plaque (from either α or β lysate), titrating in decimal dilutions and, if necessary, repeating this process until the culture is pure. From such a culture γ particles can be propagated in the same way as β particles and will give approximately the same final concentration. When γ lysate is suitably diluted and plated the particles will appear at first sight to have 'bred true', as only lytic plaques are to be seen. Prolonged search through a series of plates will nevertheless reveal an occasional solid-centre plaque. This is illustrated in Fig. 4, which is a photograph of one of two plaques found in a search of sixteen closely-sown plates: the other, when cultured, produced typical β lysate.

γ particles are inactivated by an antiserum prepared by the immunization of a rabbit with β particles and, vice versa, β particles are inactivated by antiserum prepared from γ particles. Cross-absorption tests have not been carried out.

Both types of particle have the same high resistance to heat, being able to withstand a temperature of 80° for 30 minutes.

RELATIONSHIP OF PHAGE CONCENTRATION TO THE DEVELOPMENT OF SYMBIOSIS AND LYSIS

Turbidity curves

When a lytic phage is added to an actively growing broth culture of sensitive bacteria, the subsequent increase in opacity and, to a lesser extent, the length of time which elapses before clearing occurs, is directly related to the phage-bacteria ratio. Thus a phage concentration sufficient to produce infection of most of the bacteria in the culture markedly inhibits growth so that there is little increase in turbidity before clearing starts. Where the initial phage-bacteria ratio is, say, 1:10, considerable phage development is needed to produce the necessary number of particles to infect all bacteria, and hence prior to lysis there is well-marked bacterial multiplication and an accompanying increase in optical density. In these ways γ lysate behaves as a true lytic phage (Fig. 5).

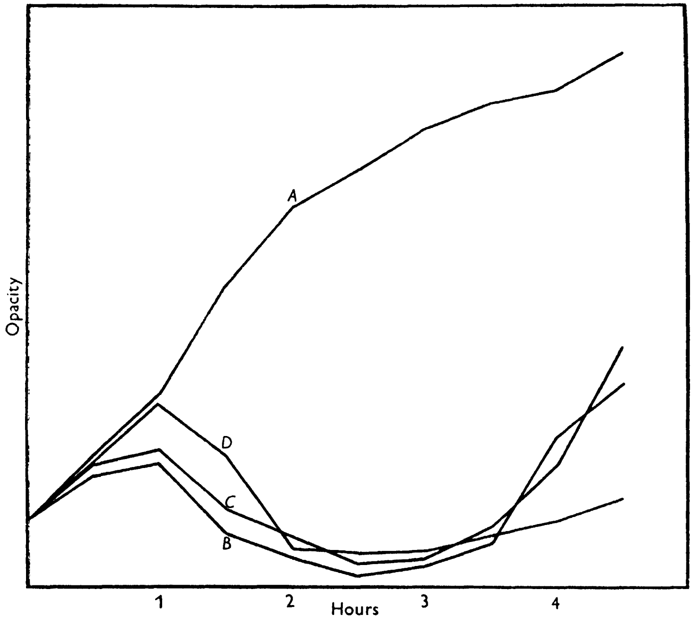

Fig. 5. Opacity curves showing the effect of exposing a growing culture of *S. typhi-murium* 1404 to different concentrations of Phage A1 (γ lysate).

A = control, no phage added to bacteria.
B = initial concentration of 10 particles per bacterium.
C = initial concentration of 1 particle per bacterium.
D = initial concentration of 1 particle per 10 bacteria.

When this experiment is carried out with β lysate a very different result is obtained (Fig. 6). Where the phage-bacteria ratio is high, no obvious lysis occurs, though the growth curve lags a little behind that of the control. Where there is approximate parity, a limited degree of lysis occurs at a time which corresponds with the completion of a cycle of lytic development; this is followed after a short interval by a steady increase in turbidity. Where the bacteria are considerably in excess of the phage particles, the curve corresponds closely to that given by a similar culture with γ particles. The explanation of this paradoxical result is in part revealed by plating suitable dilutions of the cultures and examining the colonies which develop (Table 1). In the cultures in which bacteria are exposed to a high concentration of β lysate, many of the organisms become symbiotically infected and consequently immune to lysis. In the low phage concentration, significant symbiotic infection does not occur before most of the organisms in the culture have been destroyed by lytic particles.

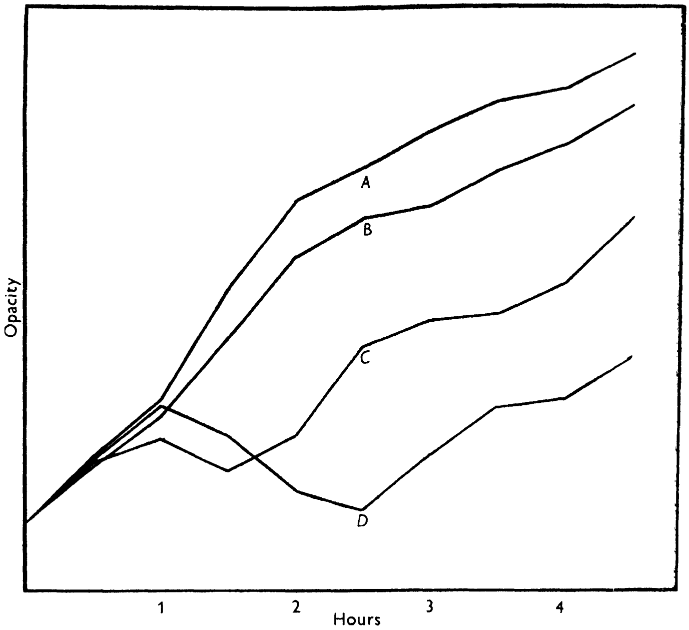

Fig. 6. Opacity curves showing the effect of exposing a growing culture of *S. typhi-murium* 1404 to different concentrations of Phage A1 (β lysate).

A = control, no phage added to bacteria.
B = initial concentration of 10 particles per bacterium.
C = initial concentration of 1 particle per bacterium.
D = initial concentration of 1 particle per 10 bacteria.

Table 1. *Relationship of the phage: bacterium ratio to the development of symbiosis in broth cultures.* Salmonella typhi-murium *and Phage A* 1 (β *lysate*)

Culture tube No.	1	2	3	4
Phage: bacterium ratio	15:1	1·5:1	1·5:10	Control
Colony count after incubation	28	22	41	58
Phage-free colonies	1	14	40	58
Phage-contaminated colonies	0	3	1	0
Symbiotically-infected colonies	27	5	0	0

Method: a series of broth cultures was prepared, containing 10^8 bacteria per ml. and β lysate in decimal dilutions giving the phage: bacterium ratio shown. After 30 min. incubation the cultures were diluted to 10^{-6}, and 0·5 ml. (equivalent to an approximate 50 bacteria in the original culture) plated. The colonies which developed were examined by the usual methods.

Impregnated agar experiments

The effect of different concentrations of phage particles on sensitive bacteria can be studied in another way by depositing a small number of organisms on the surface of a plate of nutrient agar in which a lysate has been incorporated in quantities calculated to give a known number of virus particles per ml. of medium. In such an experiment the bacterium will suffer one of several fates.

1. It may, where phage concentration is low, alight on a portion of the medium where there are no phage particles. It will then develop into a normal colony.
2. Such a colony, at first uninfected, may, as it grows, impinge on a phage particle. Partial lysis will develop, producing characteristic changes in the appearance of the colony. Colonies affected in this way will be designated 'phage-contaminated'.
3. The bacterium may come directly into contact with a particle endowed with lytic properties. This will result in its extinction. No visible colony will develop.
4. It may become symbiotically infected, in which case it will form a normal-looking but lysogenic colony.

Table 2 is a typical example of the results obtained in such an experiment carried out with β lysate. Similar findings have been obtained in many confirmatory tests carried out along the same lines. On the plate with a concentration of 10^2 particles of phage per ml. of medium, none of the colonies has come into contact with phage and all are consequently normal. With 10^3 particles about half the colonies are normal and half phage-contaminated. With 10^4 and 10^5, all are phage-contaminated.

Table 2. *Development of colonies of* Salmonella typhi-murium *on nutrient agar medium impregnated with Phage A*1 (*β lysate*)

	Plates of medium impregnated with phage								
Number of plate	1	2	3	4	5	6	7	8	9
Phage per ml. agar	10^9	10^8	10^7	10^6	10^5	10^4	10^3	10^2	nil
Total colonies	87	42	33	91	119	141	130	139	118
Colonies normal in appearance	85	42	32	0*	0	0	*c.* 50%	139	118
Colonies phage-contaminated	2	0	1	91	119	141	*c.* 50%	0	0
Characters of normal colonies	Lysogenic			No normal colony			Phage-free		

Method: *β* lysate was added to melted nutrient agar to give the required concentration of phage particles, and plates were prepared from this. After drying, each plate was flooded with 0·5 ml. of a suspension of *S. typhi-murium* 1404 estimated to contain a total of between 100 and 150 bacteria. After incubation the colonies which developed were examined by the usual methods.

* In another similar experiment one lysogenic colony was found in a plate having this phage concentration.

When there are 10^6 particles per ml., the reduction in colony numbers suggests that some of the bacteria have been destroyed by lysis, but about two-thirds have formed colonies before coming in contact with phage. At 10^7, 10^8 and 10^9, apart from an occasional phage-contaminated colony (which is probably to be explained by imperfect mixing of lysate and agar) all the bacteria deposited on the plate are either lysed or symbiotically infected, and the proportion of lysogenic colonies increases with the particle concentration in the agar. Assuming that 130 bacteria were deposited on each plate, the relative numbers which were symbiotically and lytically infected are shown in Table 3.

Table 3. *Fate of bacteria on medium impregnated with Phage A*1 (*β lysate*) (*Figures extracted from Table* 2)

	Plates of medium impregnated with phage			
Number of plate	1	2	3	4
Phage particles per ml. of agar	10^9	10^8	10^7	10^6
Colonies symbiotically infected (lysogenic)	87	42	33	0
Colonies lysed or phage-contaminated	43	88	97	130

Note: The figures of the bottom row are hypothetical, being based on the assumption that each plate was inoculated with 130 bacteria. This is the mean of the number of colonies on plates 5 to 9, Table 2.

The results of a similar experiment with γ lysate are shown in Table 4. Here the lower concentrations show colony contamination similar to that given by β lysate: the higher concentrations give increasing lysis. It is of interest to record that in the course of several experiments such as this carried out with γ lysate, on one occasion a single lysogenic colony was found. This finding agrees with the observation that rare solid-centre plaques can be found among the lytic plaques produced by γ lysate.

Table 4. *Development of colonies of* Salmonella typhi-murium *on nutrient agar medium impregnated with Phage A*1 (*γ lysate*)

	Plates of medium impregnated with phage						
Number of plate	1	2	3	4	5	6	7
Phage particles per ml. of agar	10^9	10^8	10^7	10^6	10^5	10^4	nil
Total colonies	0	2	5	38	81	64	78
Colonies normal in appearance	0	0	0	0	0	0	78
Colonies phage-contaminated	0	2	5	38	81	64	0

Method: As in Table 2. Each plate was inoculated with 0·5 ml. of a suspension of *S. typhimurium*, 1404 estimated to contain between 50 and 100 bacteria. No symbiotically infected (lysogenic) colonies developed.

These impregnated-agar experiments do no more than reproduce in a different way the phenomena which have already been revealed by the turbidity curves. The basic principles are the same. When bacteria are exposed to a high concentration of β lysate, many become symbiotically infected. As the phage concentration decreases, smaller numbers are symbiotically infected and a higher proportion—as revealed by the absence of visible colonies—are lysed. When exposed to γ particles, lysis alone takes place at all concentrations.

EVIDENCE OF MUTATION

Two distinct yet closely related patterns of mutation are revealed by these experiments: one, less common than the other, results in the formation of a comparatively stable lytic phase: the second, a rapid diphasic variation, is in constant operation whenever lysogenic bacteria are in contact with uninfected sensitive organisms.

Stable lytic mutants

The observations already recorded on the formation of stable lytic mutants require little elaboration. Occasional—in some cases very rare—γ particles are produced in a lysate formed by propagating β particles on the indicator strain. These γ particles can be recognized by the characteristic plaques they produce (Fig. 1), from which they can be grown in pure culture. Although they retain their identity indefinitely, γ particles in their turn produce very rare β mutants. This constitutes a simple two-way mutation between two phases of the virus and is important because it establishes beyond doubt the fact that mutation occurs in these phages without obvious artificial stimulus and during their normal course of development and because it provides an example of a stable lytic phage arising from a symbiotic parent.

Formation of symbiotic (α) particles by β particles

The second pattern of mutation is more obscure. Evidence of its occurrence is to be found in the symbiotically infected organisms which develop in the centre of a primarily lytic plaque; in the growth of symbiotically infected organisms in a culture containing a high initial concentration of β particles: and in the development of lysogenic colonies from normal bacteria sown on a plate containing, like the culture, a high concentration of β particles.

That β lysate contains particles with lytic properties is unequivocally demonstrated by the turbidity curve of a culture of the indicator organism exposed to an initial low phage concentration (Fig. 6). Why is this lysis replaced by symbiotic infection when the culture is exposed to the action of a high concentration of particles? Three possible explanations merit consideration:

1. As each bacterium is exposed to many phage particles, symbiosis might conceivably be related to multiple infection. However, exposure to heavy concentrations of γ particles (i.e. pure lytic particles) does not produce this result, nor can any reason be given why infection with more than one particle should act in this way. Such an hypothesis in fact appears highly improbable.
2. It might result from the synergic action of some non-particulate by-product of bacterial lysis which is present in sufficient concentration only in strong lysate. This possibility is discounted by the fact that a mixture of gradacol-filtered (i.e. phage-free) β lysate with γ particles fails to produce symbiotic infection.
3. It might be caused by the presence in β lysate of two types of particle, one lytic in nature, the other symbiotic. This theory envisages the existence of a third type of particle, the α particle, the parent type from which β and γ particles take origin.

The last of these theories fits well to the rather anomalous observations which have been recorded. There is some evidence, which will be discussed later, to suggest that the ratio of α to β particles in β lysate, though variable, may be of the order of 1:20. To state a hypothetical case, if β lysate contains about 5% of symbiotic α particles then, in the very unlikely event of 100% effective contact, only 0·5% or 1 in 200 of the bacteria will be symbiotically infected when the ratio of phage particles (all types) added to growing bacteria is 1:10 (Fig. 6, Curve *D*). This is an insignificant proportion and will not affect the turbidity of the culture for some hours. During this period the lytic β particles will multiply and soon reach a critical concentration, causing mass lysis. On the other hand, if the ratio is reversed and one bacterium is exposed to the action of a total of 10 particles (Fig. 6, Curve *B*) then, provided the maximum number of contacts takes place and also provided the α particles are capable of invading the bacteria to the exclusion of the lytic particles (an hypothesis which will be discussed later) 50% of the bacteria will be symbiotically infected. With this percentage of actively multiplying organisms, turbidity will increase with sufficient rapidity to mask partially the lysis of the remaining bacteria in the culture. Recent experiments have shown that the phenomenon of 'external balance' between lytic phage and sensitive bacterium (mentioned elsewhere) plays a part in this curve. The underlying cause of this external balance has not yet been elucidated. Its presence complicates the issue but does not in any way invalidate the conclusions which have been reached.

The results given by plating normal bacteria on agar plates impregnated with β lysate are in every way compatible with the presence of two types of particle, one symbiotic and dominant in character, the other lytic. Exposed to high concentrations of β lysate, more than half the bacteria become symbiotically infected, the remainder disappearing through lysis. As the phage concentration falls, so the chance of the bacteria coming into contact with symbiotic particles is reduced, and the number of symbiotically infected organisms (grown into colonies on the plates) decreases, while the number of lytically infected organisms (conspicuous by their absence) increases (Table 3).

The assumption that symbiotic particles can develop to the exclusion of lytic particles is a theory which is not without precedent and finds an analogy in the 'interference' phenomenon familiar in plant and animal viruses. To quote a concrete example, Hoskins (1935) has shown that the administration of neurotropic yellow fever virus to experimental animals as much as twenty-four hours after the administration of a lethal dose of viscerotropic virus will 'block' the latter and avert the fatal result. In some similar way the symbiotic particle might well be—and indeed appears to be—dominant over the lytic particle.

Finally, there is the solid-centre plaque itself, which takes origin from a single particle. As it develops, the initial lysis which gives a clear plaque is soon followed up by a resurgence of resistant lysogenic bacteria forming the solid centre. The latter can only have acquired their infection from particles derived from the one which originated the plaque and was at first lytic.

Further evidence which suggests the existence of two types of particle is to be found in the mature and immature plaques in platings of α and β lysate. Immature plaques are noticeably common in the former where every stage in development from the early one of clear pin-point lysis to the fully-developed solid-centre plaque can be seen (Fig. 2).

β lysate, on the other hand, produces plaques of great uniformity, with only a few immature ones—actually about 1 in 20 or less. A clue to the origin of these immature plaques is given by spinning a broth culture of a lysogenic strain until the supernatant fluid appears quite clear. Despite its clarity this fluid still contains many bacteria, as can readily be demonstrated by plating. If part of this be heated to kill the bacteria, and suitable volumes of both the heated and unheated portions be spread on an indicator plate and incubated, the unheated gives rise to a much larger number of immature plaques than the heated, though the latter also has its quota (Figs. 2 and 3). The explanation is simple. The bacteria in the unheated sample (which are lysogenic) have multiplied on the plate, and some of them have liberated lytic particles at a time when development of the indicator organisms on the plate was well advanced. In consequence, the plaques initiated in this way have had no opportunity to develop beyond their early stages before growth of the bacteria on the plate ceased.

If lysogenic bacteria, outwardly normal at the time when they are spread on the indicator plate, can give rise to immature plaques, it is logical to postulate that phage particles endowed with symbiotic properties could produce similar plaques, starting one stage earlier by infecting sensitive bacteria and rendering them lysogenic.

A comparison of the irregular-sized plaques formed by α lysate and the uniform plaques of β lysate strongly suggests some fundamental difference in the particles of the two lysates, which could be explained by the presence of a larger proportion of α particles in α lysate. Because of the low concentration of particles in this lysate, no experimental method of confirming this suggestion has been devised. It seems more than a coincidence that the average number of particles in β lysate which form immature plaques bears a relation to the number of bacteria in a culture which become symbiotically infected when exposed to different concentrations of this lysate.

The presence of particles with different functions—lytic or symbiotic as the case may be—in a lysate derived from a single β particle permits of no other interpretation than that mutation has occurred during the process of multiplication. This conclusion finds support in the direct evidence which the first type of mutation affords. The difference between these two mutation cycles is one of degree only. In the first, the instability is slight and mutants are rare. In the second, instability is marked, and the number of symbiotic or α mutants produced during the propagation of β particles is considerable. A reversion from β to α is therefore constantly taking place.

Mutation of α particles in lysogenic bacteria

It is now possible to turn to a consideration of the *fons et origo*, the lysogenic culture from which, when it is in a state of active growth, a steady stream of phage particles is liberated.

Reference has already been made to Lwoff's work on the lysogenicity of *B. megatherium* and to his theory that in its latent phase the phage is in a state of arrested development (probacteriophage) from which it emerges when some modification takes place in the metabolism of the host bacterium. Such modifications can be produced at will by ultraviolet radiation, by X-rays and by certain chemicals, and are affected by the composition of the medium in which the bacteria are suspended or grown (Lwoff, 1951). Although Lwoff states that alterations in bacterial metabolism are without doubt capable of stimulating the onward development of probacteriophage, he admits that he cannot rule out the possibility that some modification of the probacteriophage is responsible.

In the case of *S. typhi-murium* and its 'A' phages it has been shown that, in the association of free particles with the indicator strain, the establishment of symbiotic infection or alternatively the initiation of the lytic cycle of development is determined by the characters of the particle and not by those of the bacterium. If a bacterium is penetrated by a particle with lytic propensities it undergoes lysis, if by a particle with symbiotic propensities, it becomes symbiotically infected. It might be argued that, while admitting free particles with these different characters do occur, they differ only in their degree of maturity. Such a contention is difficult to maintain. The fact that symbiotic particles can exist in the free state side by side with lytic particles, that they are dominant over the lytic particles in establishing themselves in the bacterium, that they can live harmlessly in the host bacterium and multiply 'in step' with it, seems to establish for them an unassailable claim to the status of a distinctive and mature phase in the life cycle of the phage. It is, of course, very possible that the starting point in the disharmony which brings about the lytic incident within the lysogenic bacterium is some variation in the bacterial metabolism and some consequent modification of the chemical environment of the phage. This, however, provides no reason for assuming that the change which takes place in the phage is the resumption of arrested development. On the contrary, knowing that the symbiotic particle is, in the free state, distinct from the lytic particle and can itself arise by mutation, there appears good reason to conclude that the immediate cause of lysis is a reverse mutation in which a lytic particle is thrown off by the symbiont and becomes incorporated in a daughter bacterium. In terms of this theory, the cycle has been completed and the symbiont which arose from the mutation of a lytic particle has in its turn undergone mutation to produce lytic particles.

CYCLE OF MUTATION

It now becomes possible to give a fairly complete account of the cycle of mutation of phage A.

The α particle, securely established in the bacterium, and assured of all its requirements for maintenance and multiplication as long as the latter remains alive, constitutes the reservoir of infection. Its contribution to the common good in the symbiosis is the protection of the bacterium against the inroads of related lytic phages other than mutants derived directly from itself. Apart from such internal lytic incidents, the union of phage and bacterium appears to be permanent, although it is impossible to assert that dissociation does not occur under certain conditions.

With each division of the bacterium the phage duplicates itself so that each daughter bacillus remains phage-infected.

From time to time one of the particles produced by division is a mutant with lytic properties. This mutant undergoes rapid multiplication in the daughter bacterium which receives it, and produces α, β and γ particles, all of which are liberated when the bacterium bursts. In suitable conditions these free particles remain viable for long periods of time and undergo further development when they come in contact with a sensitive host bacterium. The α particle in such circumstances establishes symbiotic infection. The β particle multiplies in its host by the lytic cycle, producing mainly β particles but with a high proportion (up to 5%) of α mutants and rare γ mutants. The γ particle also multiplies by the lytic cycle, producing almost exclusively γ particles but also very rare β mutants. The cycle of variation is diagramatically depicted in Fig. 7.

MUTATION IN OTHER TYPES OF PHAGE

Mutation is by no means confined to the A phages, but it is perhaps most easily observed in them because of the size of the plaque and the ease with which a high concentration of particles can be obtained. Although the plaques of the B phages of *S. typhi-murium* are replicas in

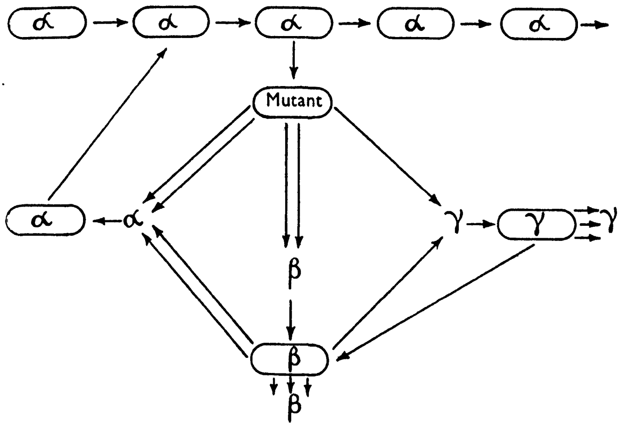

Fig. 7. Diagrammatic representation of the mutation which occurs in Phage A.
α = symbiotic particle.
β = unstable lytic particle.
γ = stable lytic particle.
One arrow signifies 'rare'.
Two arrows signify 'moderate numbers'.
Three arrows signify 'numerous'.

miniature of those of the A phages, they are more difficult to investigate because, with the methods and media used, they do not multiply fast enough to give clearing in a liquid culture. Lysis can, of course, be seen in the plaque itself. Stable lytic variants have been recovered from three B phages, and one of these is identical with an 'O' phage kindly supplied by Dr Felix some years ago. There is little doubt that if this matter were pursued actively—so far it has not been possible to do so—all of these phages would yield stable lytic variants.

The mutation observed by Burnet & Lush (1936) in a phage active against a strain of *Staphylococcus albus* is almost identical with that which occurs in the A phages. These workers describe exactly the solid-centre plaques, and note that this original phage produces 'resistant forms' with remarkable frequency—actually in some 10 to 20% of effective contacts with the indicator coccus. These resistant forms were found to be lysogenic. As the culture 'aged', mutants were formed which gave clear plaques, which did not show granules as in the case of the A phages. Unfortunately this observation does not appear to have been carried any further and no explanation of the phenomena is attempted.

A search of the literature suggests that others have encountered mutation from the symbiotic to the lytic phase without appreciating its significance. Thus, some plates in papers by Fisk (1942*a* & *b*) illustrate lysis caused by phage which was primarily symbiotic. Felix & Callow (1943), detailing the sources from which anti-Vi phages for *S. paratyphi* B were isolated (these are of course lytic phages), state that 'five were from lysogenic rough variants'. Observations by Warner (1950), Scholtens (1950) and Nicolle, Hamon & Edlinger (1951) suggest similar findings, and there are without doubt many others.

An important difference between some of these findings and those described in relation to phage A is that no solid-centre plaque is mentioned, which implies that there is little or no tendency towards reversion from the lytic to the symbiotic type. Mutants with these properties would not give either the turbidity curves or the results on impregnated agar which are so characteristic of phage A. It may be an intrinsic character of these phages that they produce only stable lytic variants. But before such a conclusion is reached it is necessary to find out if the indicator strain used corresponds in every way, except in lysogenicity, to the strain from which the phage was derived. It is well known that phage from a lysogenic strain may have a purely lytic action on another species of bacterium. The original observation of Lisbonne and Carrère (1922) of a phage derived from a strain of *E. coli* which has a powerful lytic action on Shiga's bacillus is an example of this, and others which need not be detailed come within the writer's own experience. It is not outside the bounds of probability that the findings mentioned above and also some of the conflicting opinions which have been formed regarding symbiotic bacteriophage may have arisen from experiments carried out with an improperly attuned indicator.

If experience confirms that stable lytic variants can be derived from all symbiotic phages, it is more than probable that the converse is also true and that all lytic phages spring from symbiotic parents. This calls for a revision of many current ideas and indicates lines along which a rational classification of phages can be reached.

SIGNIFICANCE OF THE CYCLE OF MUTATION

The recognition of this cycle of mutation widens the approach to the problems of phage multiplication. It makes clear the fact that the lytic or virulent phase is but one aspect and that attention must also be directed to the parent symbiotic particle, to its relation to the bacterium, its mode of division, and the factors which govern the birth of mutants. In this paper it is not proposed to do more than mention some of the outstanding points which await investigation.

SOME OBSERVATIONS ON THE SYMBIOTIC PARTICLE

No direct evidence exists as to the location of the symbiotic particle in the bacterium. Tentative conclusions on this subject can be reached by inference only. One fact can, however, be stated categorically. Symbiotic particles produce no reaction in the bacterium resembling that which follows infection with a lytic particle: the chromatin (nucleus) of the bacterium remains in a compact mass and is not in any way dispersed throughout the cytoplasm.

Certain findings suggest that the particle fuses with some element within the bacterium and in doing so surrenders permanently its capacity for independent existence. All attempts to recover symbiotic particles by disrupting the infected bacterium have been unsuccessful: active phage can emerge only by rebirth through the medium of a lytic mutant. Further evidence of the close integration of phage and bacterium is afforded by the fact that the former acquires the thermal death point of the latter. Phage A in the free form resists a temperature of 80°. In union with the bacterium it is destroyed, in company with its host, by exposure to 55°. Conversely, it has been shown by den Dooren de Jong (1931) that a symbiotic phage of *B. megatherium*, normally inactivated by exposure to 75° for 5 minutes can, when present in the spores, resist 100° for the same length of time.

A notable character of symbiotic phage is its controlled and periodic division which occurs in such a way that infection is passed on without fail to every daughter bacterium. It was recently suggested (Leading Article, *Lancet*, 1951) that this certainty of infection might be attributable to the presence of several phage particles scattered throughout the cytoplasm of the organism. As lysis does not occur if a symbiotic particle is present in a bacterium, this seems an unlikely idea. It appears more probable that a single particle is related to some structure in the bacterium which invariably takes part in the cell division and divides in unison with it.

All these considerations point to the conclusion that the phage becomes closely associated, if not actually fused, with the nucleus of the bacterium, a conclusion which receives further support from the similarity of chemical structure between phage and nucleus, and from the fact that viruses of all kinds are closely associated with the nuclei of the cells in which they develop. In such a situation, too, the symbiotic phage is well placed to block invasion by the lytic variants.

Even in this apparently subordinate existence the phage in some way retains its identity so that, when the nucleus divides, the phage also duplicates itself and passes on into each daughter cell. If, either because of some external stimulus or because of its inherent instability, one of the daughter particles has the characters of a lytic mutant, this finds itself ready set in its optimum environment and proceeds to dominate the metabolism of its host bacterium, reproducing the chain of events which characterize the lytic cycle of development.

STATUS OF LYTIC PHAGES

With few exceptions, all the well-known phages are 'lytic variants' and, if the conclusions which have just been reached are accepted, must have taken origin from some symbiotic parent which may reside either in other races of the same bacterium or, as happens for example with the Shiga phage of Lisbonne and Carrère, in a different species of organism. The fact that these lytic phages are 'end-products' in no way detracts from their importance. They represent the virulent or destructive phase of the phage, and correspond to the disease-producing phase of the plant and animal virus. A study of the metabolism and of the factors concerned in the reproduction of such phages cannot fail to throw light on analogous processes in these other viruses and may indeed uncover the means by which their action may be counteracted.

The lytic variants, however, can play no more than a minor role in maintaining the continued existence of the strain during what may be termed endemic periods. A parasite which invariably destroys its host has an insecure future and, no matter how high its infectivity, depends on recurring contacts with new hosts for its survival. We are so accustomed to thinking in terms of artificial cultures and the conditions of the laboratory that we are apt to forget the minute size of both phage and bacterium, and, in consequence, the relative immensity of the spaces which separate them. It may be argued that in certain environments, such as in sewage or in areas which are constantly being fouled by faeces, the concentration of both phage particles and bacteria must be sufficiently high to ensure these frequent contacts. But let any worker attempt to isolate some specific phage from material of this kind, using the most favourable technique he can devise, and he will realise from his failures how relatively rare such phage particles are. Again, though free phage particles can be kept viable for long periods under laboratory conditions, it is unlikely that they will have the same longevity in their natural surroundings. They may well be acceptable food morsels to insusceptible bacteria. Taking all these points into consideration, the chances in favour of the indefinite survival of a phage would be poor if it depended solely on the lytic cycle. However, the steady stream of particles from the original source, together with, in certain cases at least, new and continuing infections set up by symbiotic mutants arising during the lytic cycle of development, ensure that the strain does not become extinct.

If phages and other viruses be considered from a teleological standpoint the lytic or virulent variant must in the light of these conclusions be regarded, not as the final stage in the development of the parasite, but as a ruthless expedient which has evolved to extend the domain and doubly ensure the continuity of the species.

SOME SPECULATIVE APPLICATIONS OF THIS CYCLE OF MUTATION

While there are no grounds for assuming that all viruses have a life cycle similar to the one which has been described, yet it is interesting to examine some of the problems of virus disease in the light of these findings. In those infections in which there is only one host, such a cycle provides a simple—perhaps too simple—explanation of the way in which the virus maintains itself in the inter-epidemic period. A latent symbiotic infection which, at the onset of an epidemic, is stirred up by some stimulus acting in the same way as ultra-violet radiation does on lysogenic *B. megatherium*, accounts in a more convincing manner for the survival of the virus than does, say, a chain of subclinical infections. Where two hosts are involved, the possibilities are even more intriguing. In yellow fever, for example, the virus-carrying mosquito is otherwise normal and lives as long as if it were uninfected. Such an insect might well suffer from symbiotic infection of the cells of the salivary gland and pass on to susceptible mammalian hosts the lytic or virulent particles emanating from this source which, in due course, would infect the liver cells. In this selected environment the particles might multiply by the lytic cycle, liberating, as in the case of the phages, large quantities of lytic particles and limited numbers of symbiotic particles. Thus direct infection of fresh mammalian hosts could be (and is) effected with minute quantities of blood, while relatively large quantities would be (and are) needed to infect a clean mosquito. In many of the virus diseases of plants, the vector is one of the *Aphides* and it has but recently been affirmed (Kennedy, 1951) that purely mechanical transmission is unlikely. Here is an alternative explanation which merits consideration.

Problems of immunity are also raised. Is it possible that the life-long protection conferred by an attack of certain virus diseases is primarily attributable, not to residual antibodies, but to a lasting symbiotic infection—the incorporation in the nucleus of the susceptible cells of some permanent element which for ever debars the entrance of its lytic counterpart? Is the persistence of humoral antibodies after certain infections caused by the recurring stimulation of the defence mechanism by the trickle of lytic mutants from the infected cells—an arrangement which ensures both immunity and in most cases non-infectivity? Conversely, is the lack of immunity to the common cold, of which we are too well aware, due to the fact that this virus fails to establish symbiotic infection in the human being? If so, where is the symbiotic infection, if any, to be found?

When other workers confirm and no doubt amplify this mutation hypothesis insofar as it refers to bacterial viruses, and extend its application to plant and animal viruses, it can be hoped that a new direction will be given to the more specialized methods of research which in the past have been mainly concerned with a somewhat restricted aspect of the virus problem.

Acknowledgments

Tables 2 and 3 (slightly modified) and Figs. 1 and 2 are reproduced by courtesy of the Editor of the *Journal of Pathology and Bacteriology*. Figs. 4, 5 and 6 are reproduced by courtesy of the Editor of *Nature*.

REFERENCES

BOYD, J. S. K. (1949). Morphological changes in bacteriophage-infected organisms as revealed by phase-contrast illumination. *J. Path. Bact.*, **61**, 127.

BOYD, J. S. K. (1950). The symbiotic bacteriophages of *Salmonella typhi-murium*. *J. Path. Bact.*, **62**, 501.

BOYD, J. S. K. (1951*a*). Observations on the relationship symbiotic and lytic bacteriophage. *J. Path. Bact.*, **63**, 445.

BOYD, J. S. K. (1951*b*). Mutation in a bacterial virus. *Nature*, **168**, 994.

BURNET, F. M. (1930). Bacteriophage reactions of Flexner dysentery strains. *J. Path. Bact.*, **33**, 637.

BURNET, F. M. (1932). Lysogenicity as a normal function of certain Salmonella strains. *J. Path. Bact.*, **35**, 851.

BURNET, F. M. & LUSH, DORA (1936). Induced lysogenicity and mutation of bacteriophage within lysogenic bacteria. *Aust. J. Exp. Biol. Med. Sci.*, **14**, 27.

BURNET, F. M. & MCKIE, MARGOT (1929). Observations on a permanently lysogenic strain of *B. enteritidis gaertner*. *Aust. J. Exp. Biol. Med. Sci.*, **6**, 277.

DEN DOOREN DE JONG, L. E. (1931). Studien über Bakteriophagie. 1. Ueber *Bac. megatherium* und den darin anwesenden Bakteriophagen. *Zbl. Bakt.*, **120**, 1.

FELIX, A. & CALLOW, BESSIE, R. (1943). Typing of paratyphi B. bacilli by means of Vi bacteriophage. *Brit. Med. J.*, *ii*, 127.

FISK, R. T. (1942*a*). Occurrence of bacteriophage carriers among strains of *Staphylococcus aureus*. *J. Infect. Dis.*, **71**, 153.

FISK, R. T. (1942*b*). Identification of *Staphylococcus aureus* strains by means of bacteriophage. *J. Infect. Dis.*, **71**, 161.

HOSKINS, M. (1935). A protective action of neurotropic against viscerotropic yellow fever virus in *Macacus rhesus*. *Amer. J. Trop. Med.*, **15**, 675.

KENNEDY, J. S. (1951). A biological approach to plant viruses. *Nature*, **168**, 890.

Leading article (1951). Lysogenicity in bacteriophage strains. *Lancet*, *ii*, 977.

LISBONNE, M. & CARR`RE, L. (1922). Antagonisme microbien et lyse transmissible du bacille de Shiga. *C. R. Soc. Biol., Paris*, **86**, 569.

LURIA, S. E. & HUMAN, MARY L. (1950). Chromatin straining of bacteria during bacteriophage infection. *J. Bact.*, **59**, 551.

LWOFF, A. (1951). Conditions de l'efficacité inductrice de rayonnement ultraviolet chez une bactérie lysogène. *Ann. Inst. Pasteur*, **81**, 370.

LWOFF, A. & GUTMANN, ANTOINETTE (1950). Recherches sur un *Bacillus megatherium* lysogène. *Ann. Inst. Pasteur*, **78**, 711.

LWOFF, A., SIMINOVITCH, L. & KJELDGAARD, N. (1950). Induction de la production de bactériophages chez une bactérie lysogène. *Ann. Inst. Pasteur*, **79**, 815.

NICOLLE, P., HAMON, Y. & EDLINGER, E. (1951). La lysogénéité des différents types de *Salmonella paratyphi* B. *Ann. Inst. Pasteur*, **80**, 479.

SCHOLTENS, R. T. (1950). Lysogenicity as a tool for bacteriophage typing of *Salmonella paratyphi* B. *Antonie van Leeuwenhoek*, **16**, 256.

WARNER, P. T. J. C. P. (1950). The isolation of the bacteriophages of *Ps. pyocyanea*. *Brit. J. Exp. Path.*, **31**, 112.

WEIGLE, J. J. & DELBRÜCK, M. (1951). Mutual exclusion between an infecting phage and a carried phage. *J. Bact.*, **62**, 301.

DISCUSSION

A. LWOFF: In order to avoid confusion, it would perhaps be worth while to abandon the expression 'symbiotic phage' for the phage of lysogenic systems. The expression 'lysogenic phage' is also somewhat misleading.

It seems useful and I would like to propose again

(*a*) to refer to the phage particles produced by lysogenic systems as 'temperate phage', except for virulent mutants—temperate being opposed to virulent,

(*b*) not to use the term 'symbiotic phage' for the non-infective and non-pathogenic particle—or concept—responsible for the maintenance of lysogenicity in lysogenic bacteria. This particle lacks many of the properties of bacteriophage.

Prophage has been defined as the form in which lysogenic bacteria perpetuate the property to produce phage. This would be exactly the definition of Dr Boyd's 'symbiotic phage'. An agreement has to be reached on these unfortunate problems of nomenclature. As the term 'prophage', whether good or not, is now in general use and as 'symbiotic phage' is not a phage, I prefer to maintain 'prophage'.

J. S. K. BOYD: I agree that the expression 'lysogenic phage' is misleading.

(*a*) Dr Lwoff's proposal is to use the term 'temperate phage' for the free phage particles produced by a lysogenic system. According to my conception, this lysate contains particles of three types, one capable of producing symbiotic infection, one of developing by the lytic cycle, and the third being a relatively stable lytic mutant which Dr Lwoff regards as 'virulent'. The majority of the particles in this lysate, when allowed access to sensitive bacteria in low concentration, produce the same ultimate lysis as 'virulent' particles. The 'temperate' nature of the lysate, as I see it, lies in the fact that symbiotically infected bacteria are produced by it. The conception of different types of particle gives a clearer expression to my idea. This would be obscured by the term 'temperate phage'.

(*b*) This is mainly a question of nomenclature, and Dr Lwoff and I are fundamentally in agreement. A bacteriophage is something which eats away a bacterium, so that symbiotic phage is, if the radical meaning of the words be applied, a contradiction in terms. The difficulty might be solved by calling it 'symbiotic virus'. However, I realize that the term 'prophage' (with the definition which Dr Lwoff has given in his paper) may—like lysogenic phage—have come to stay.

A. LWOFF: It seems clear from Dr. Boyd's data that the fate of a *Salmonella typhi-murium* may depend on whether it is infected either by one or by many bacteriophage particles. In this case, bacterial immunity would not be the result of bacterial mutation but the consequence of a multiple infection producing a modification of the bacterial metabolism preventing phage development and allowing the infected bacterium to multiply and to give rise to a lysogenic clone. We know that when prophage develops into phage it is because something has been modified in the lysogenic system, either the structure or properties of prophage or the properties of the bacterium. If I understand Dr Boyd's conception correctly we have to assume that prophage in *S. typhi-murium* is in the α-form. It would have to mutate to β form in order to develop. Then, in the course of development, β-phage particles would be produced and some α mutants. Only bacteria infected with these α-mutants would give rise to lysogenic clones. This conception could correspond to the facts but has the disadvantage of being impossible either to prove or to disprove.

J. S. K. BOYD: Dr Lwoff is right in saying that there are at present no experimental data to decide between the two theories of lysogenic infection in this particular system—i.e. whether it arises from infection with numerous particles or from infection with one particle having special properties.

My reason for suggesting the latter is that no such lysogenic infection results when *S. typhi-murium* is exposed to multiple infection with the stable lytic—i.e. γ mutant.

I note that Dr Lwoff agrees with my conclusion that, in the case of this particular system, the development of lysogenicity seems to be a function of the phage and not of the bacterium.

E. S. ANDERSON: The validity of Brigadier Boyd's claim that there are two types of particle involved in the normal life cycle of a bacteriophage capable of producing lysogenicity in an organism rests largely on experiments with phage-impregnated agar such as that summarized in Table 3 of his paper. It is interesting to calculate the actual distribution of phage particles to which the bacterial inocula are exposed, assuming that the only effective particles lie near the surface of the agar. When the concentration of phage is 10^9 per ml., the particles, if uniformly distributed, will lie 10μ apart. Brigadier Boyd has stated in introducing his paper that they are about 35 mμ in diameter, so that in comparison with a bacterial cell of about $3{\cdot}0 \times 0{\cdot}5\mu$, they can be regarded as having position but no magnitude. If 'symbiotic' particles exist in this phage with their postulated frequency of 1:20 of the 'lytic' particles, an organism would have to sustain an average of at least twenty contacts with phage particles in order to encounter one of the hypothetical 'symbiotic' particles, and of course Brigadier Boyd's further postulate would then be required that the 'symbiotic' particles must have an ascendancy over the 'lytic' particles as far as entering the cell is concerned.

If we assume that the number 43 in the bottom row of column 1 of Table 3 largely represents organisms which have disappeared as a result of immediate infection with 'lytic' particles, and that the 87 lysogenic colonies which survived represented bacteria which had immediately become 'symbiotically' infected, the unlikely conclusions emerge that the 130 bacteria deposited on the plate had not only immediately achieved 100% effective contact with one or more phage particles but that two-thirds of these bacteria had encountered an average of at least 20 particles each, the minimum average contact required to ensure an encounter with one 'symbiotic' particle. Bearing in mind the distance between phage particles at the surface of the agar in the concentrations of phage used, and the size of the organisms, it is obvious that this is unlikely to be a correct interpretation of the experiment.

A more accurate estimate of the likelihood of contact between phage particles and bacteria can be achieved by assuming that the particles conform to a Poisson distribution. There is no information as to the effective depth to which the bacterial cell can encounter phage particles but it seems not unreasonable to assume that it is not greater than the length of the bacterial cell, so that the only operative phage-particles will be those lying in a surface layer of agar about 3μ thick. When the concentration of phage is 10^9 per ml., a lamina of this thickness 1 sq. cm. in area will contain 3×10^5 phage particles. If the effective area of the bacterium be liberally taken as 5×10^{-8} sq. cm., then the average number of particles underlying a bacterial cell

$$\begin{aligned} &= \text{density of particles} \times \text{effective area of cell} \\ &= 3 \times 10^5 \times 5 \times 10^{-8} \\ &= 15 \times 10^{-3}. \end{aligned}$$

Therefore, the fraction of bacteria having no underlying particles

$$\begin{aligned} &= e^{-15 \times 10^{-3}} \\ &= 0{\cdot}985 \text{ approx.} \end{aligned}$$

that is, 98·5% of cells will not encounter phage particles at all. If 'symbiotic' particles occur with the postulated frequency of 1:20 of 'lytic' particles, then by the same reasoning, the fraction of bacteria having no underlying 'symbiotic' particles

$$\begin{aligned} &= e^{-7{\cdot}5 \times 10^{-4}} \\ &= 0{\cdot}9992 \text{ approx.} \end{aligned}$$

that is, 99·92% of cells will not encounter 'symbiotic' phage particles when they alight on the agar surface.

Turning again to column 1 of Table 3 of Brigadier Boyd's paper it can be seen that if the particles are assumed to have a Poisson distribution, which is likely, the chances are that only 1·5% of the bacteria deposited on the plate had alighted on phage particles of any sort and that no more than 0·08% had a chance of hitting a 'symbiotic' particle. The chances against 87 out of 130 bacteria alighting on 'symbiotic' particles are astronomical. A much more likely explanation would seem to be that most bacteria alight in spaces between phage particles, and that contacts with the latter occur mainly by the extension of growing colonies. When the phage concentration is 10^9 per ml. of medium the spacing of particles on the surface will ensure effective contact with, and multiplication of, phage early in the life of most colonies, so that high concentrations of phage will be released into relatively small aggregates of organisms; this seems to be the condition required for the establishment of 'symbiotic' infection and the surviving organisms of the microcolonies will grow uninterruptedly to form lysogenic colonies.

In columns 2 and 3 of Table 3 where the concentrations of phage are 10^8 and 10^7 per ml. the application of the foregoing arguments makes the acceptance of Brigadier Boyd's theory even more difficult.

The greater the spacing between phage particles on the agar surface, the larger will be the bacterial colonies before a phage particle is encountered, and once the colonies are large enough the evidence they will show of contact with phage will be that which Brigadier Boyd describes as 'phage contamination'. This is because the degree of growth of the colony by the time particles are released into it in relatively high concentration is already at or near its maximum so that a secondary growth of lysogenically infected organisms cannot occur. Indeed there are still many susceptible organisms of the original colony present which are not lysed by the phage because the optimal growth phase has passed. As the phage concentration in the medium diminishes and the spacing between particles becomes wider, so the expected time of contamination of colonies with phage rises to a maximum, beyond which the proportion of phage-contaminated to phage-free colonies drops as the spacing makes it progressively more unlikely that a colony will encounter a phage particle at all during the course of its growth. Rows 3 and 5 of columns 4, 5, 6, 7, 8 and 9 of Table 2 of Brigadier Boyd's paper illustrate this very well, and he himself has made similar observations (Boyd, *J. Path. Bact.*, 1951, **63**, 445).

It can be seen, therefore, that all the findings described by Brigadier Boyd in the experiments with phage-impregnated agar can be most satisfactorily explained on the following bases: even with a phage concentration of 10^9 particles per ml. of medium the initial likelihood of an organism encountering a phage particle when it alights on the medium is low, and of sustaining a twentyfold multiplicity of infection extremely low; all the phenomena found are the end result of the infection with phage of bacterial colonies of varying age. There is no evidence to support the claim that there are 'lytic' and 'symbiotic' particles which are functionally different.

If we turn now to the consideration of the plaques produced by the 'α-lysate' described by Brigadier Boyd as a further support for his hypothesis of functional duality in the particles of phages capable of producing lysogenicity in bacteria, it is apparent that the great variation in size of plaque again does not support this theory. The solid-centre plaque can be dismissed as being derived from particles of phage free in the medium. But the plaques showing a range of size from pinpoint upwards can be explained on two alternative hypotheses, both of which may be operative. First, in an unheated supernatant there are lysogenic bacteria which when deposited on the indicator strain will, after an unpredictable interval, initiate plaques. Such plaques, however, could as well stem from a prophage, as described by Lwoff in his contribution to this symposium and in his previous work quoted therein, as from the emergence of a 'lytic' mutant from a 'symbiotic' particle. The experiment will not disdistinguish between the two hypotheses. Second, it must be remembered that an 'α-lysate' placed on an indicator strain contains quantities of bacterial receptors and bacterial debris, all of which may have adsorbed phage particles. It has not been demonstrated conclusively in the case of phages capable of entering into a lysogenic relationship with their bacterial host that such adsorbed phage particles cannot become free to attack growing organisms. If such a release is possible, it would also occur in a random fashion and would, therefore, produce a picture exactly similar to that shown in figure 2. There is nothing here, therefore, to support the claim of phage particles which are functionally different in a 'lytic' or 'symbiotic' sense.

What is obvious from Brigadier Boyd's work is that the presentation of growing organisms with a high concentration of phage particles will produce a high proportion of lysogenically infected organisms. I have also observed this phenomenon, but I think it is more satisfactorily explained on the basis of multiple infection of organisms with phage particles and consequent interference with phage multiplication in the cell. In a critical examination of the experiments described in Brigadier Boyd's paper it is not possible to find supporting evidence for the presence of 'symbiotic'⟷'lytic' phase variation as an essential part of the life cycle of the 'lysogenic' phages of *S. typhi-murium*.

J. S. K. BOYD: Dr Anderson's mathematical calculations, which I confess I had not worked out, are quite irrelevant, as they are based on the erroneous assumption that the bacteria and phage particles are stationary throughout the experiment. This, however, is not the case. The bacteria are flooded on to the agar surfaces in 0·5 c.c. of saline, which makes an obvious wet film and does not dry for some minutes. In this film the motile bacteria move about actively, as can readily be confirmed by direct observation. I doubt if any mathematician could calculate the probable number of contacts of bacteria and phage in these circumstances with any degree of accuracy, but it seems reasonable to postulate, as I did, that it bears some relation to phage concentration. The impregnated agar experiment differs from the broth culture experiment (Table I) only in that, while no phage: bacterium ratio can be calculated, the fate of the individual bacteria can be followed.

Dr Anderson has overlooked the significance of the 'missing' colonies (Table 2, plates 1, 2, 3, 4) which I attribute to 'direct hits', i.e. to infection of the original organism, before it has had a chance to divide, with a lytic particle. 'Near misses,' where the bacterium, now stationary, divides a few times before one of the daughter bacteria comes in contact with a phage particle, will inevitably produce a 'phage-contaminated' colony, though, if the 'contamination' occurs at an early stage in the colony's growth the appearance of the colony will be normal. When the one infected bacterium bursts and liberates its 100 or more particles, the adjoining organisms in the small colony will de drenched in these particles. Whether symbiotic infection of a bacterium is the result of multiple infection with phage particles (as Dr. Anderson later suggests) or of infection with a 'symbiotic' particle (as I contend) the result will be the same. One or more of the hitherto normal bacteria will become symbiotically infected and phage-resistant and will multiply to form a colony. 'Missing' colonies must, therefore, be attributable to the elimination of the original organism by a direct hit from a lytic particle. It is vaguely possible that in a starting colony where two or even four organisms are present, all might be more or less simultaneously infected by lytic particles before the first of them bursts. This, however, is no more than a modification of the direct hit. If multiplication of the bacterium has passed more than one or two divisions before a 'hit' occurs, some form of colony is bound to develop.

Dr Anderson's conclusion is that all the phenomena found in the phage-impregnated agar experiment are the result of the infection with phage of bacterial colonies of varying age. In other words, he regards the lysogenic colonies on the plates with the phage concentration of 10^9, 10^8 and 10^7 as 'near misses'. A glance at Tables 2 and 3 will show that this implies that, as the phage concentration increases, the number of 'direct hits' falls and the number of 'near misses' rises, a conclusion which is manifestly absurd. The higher the phage concentration, the greater will be the number of 'direct hits'. The increasing number of lysogenic colonies on the 10^7, 10^8 and 10^9 plates, together with the decreasing number of missing colonies, can only be explained by some form of 'direct hit' which gives symbiotic infection instead of lysis.

Turning to Dr Anderson's second criticism, my observations on the plaques formed by α lysate were not aimed at the differentiation between 'prophage' and 'symbiotic particle'. Lwoff regards 'prophage' as a normal phage particle in a state of arrested development because the bacterium is resistant or *inapte*. The impregnated agar experiment proves beyond any reasonable doubt, as pointed out in my paper, that the development of symbiotic infection in the *typhi-murium* phage-bacterium complex is a function of the phage and not of the bacterium and hence I postulate the existence of a mature symbiotic particle. The α lysate observations were not designed to 'distinguish between the two hypotheses' and I have at no time suggested that they do.

Dr Anderson states that α lysate contains bacterial receptors and bacterial debris and presumably implies that β lysate does not; hence the difference in plaque pattern. In fact, as β lysate contains the debris of 1 to 2×10^8 bacteria per ml., it is much richer in these end-products than α lysate, which contains the debris of a relatively small number of bacteria. His original—if unsubstantiated—theory that adsorbed lysogenic particles may be released in a random fashion from the bacterial host (I presume he means the debris of lysed bacterial hosts, for nothing else is present in the lysate used for Fig 2 to which he refers) therefore applies equally to α and β lysates and has no bearing on the argument.

Dr Anderson, for unstated reasons, concludes that the development of lysogenicity is to be explained on the basis of multiple infection of the organism with phage particles. This is the first of the hypotheses which I put forward in my paper and indeed when I first observed the phenomena I myself favoured this explanation. I found it untenable when I performed the experiment set out in Table 4 (many times repeated, both with this and other lytic phages). Multiple infection with lytic (γ), particles does *not* produce lysogenesis. Dr Anderson, in his 'critical examination of the experiments' has either failed to appreciate the significance of this point or conveniently ignores it.

I am afraid there is nothing in Dr Anderson's criticisms which causes me to reconsider my conclusions.

LOIS DICKINSON: We have found similar variation in the phages of a lysogenic strain of *Ps. pyocyanea*. The filtrate, containing phages Pa and Pb, from a strain of *Ps. pyocyanea* (isolated from a war wound) was passaged in a mouse together with an indicator strain C7. The strain, C7X, isolated from the mouse was iridescent and lysogenic. C7X filtrate, assayed on indicator culture C10, produced two types of plaques, one with a lytic zone surrounding a central area of bacterial growth and clearly visible at 18 hr. (P18) and the other which was much larger, hazy and only clearly seen after 48 hr. incubation of the assay plate (P48). P18, especially when passaged on C10, gave almost pure P18 type plaques but very rarely gave a large, hazy type variant. P48 gave rise to varying proportions of P18 and P48 when grown with C10. One preparation of P48 gave a lysogenic culture which produced the P18 variant during growth; a filtrate of this culture also gave the P18 variant when grown on C10. P18 = Pa, serologically and by cross resistance tests. There was little relationship between P18 and P48, but some similarity between P48 and the original Pb. Both P18 and P48 had latent periods of 1½ hrs. under our conditions, and the burst was inhibited by 2:7-bis-(2-dihydroglyoxalinyl)-9-phenyl-phenanthridine trihydrochloride. This compound had been shown to act early in the reproductive cycle of lytic phage, but to have no effect on phage production by lysogenic *cultures*. Cultures resulting from the growth of P18 on C10 contained completely sensitive cells and cells resistant to P18 but still sensitive to P48 and Pb. Cultures resulting from the action of P48 on C10 gave lysogenic bacteria, resistant to all phages. Pb phage, giving lytic type plaques, but which is probably related to P48 in view of the cross reactions, gave rise to cultures resistant to itself and P48, but still sensitive to P18; however all such cultures were apparently lysogenic. Each colony tested produced phage and the cultures were not inhibited by the phenanthridine (unlike lytic cultures) although the plaques produced were still typically lytic ones.

J. S. K. BOYD: Dr Dickinson's findings bear some resemblance to the phenomena I have described, in that certain of her phage types produce two types of plaque. It is, however, rather unusual that phages so closely related as these appear to be should be serologically dissimilar. The possibility that Pa and Pb are different types of phage should not be overlooked.

F. C. BAWDEN: Dr Boyd concludes his paper by saying that when his mutation hypothesis has been extended to plant and animal viruses it can be hoped that a new direction will be given to research. The 'when' with plant viruses was about 20 years ago, for at least this time had elapsed since it was realized that plant viruses can undergo striking changes in virulence and that the genetic constitution and physiological condition of a host plant can also strikingly affect the type of symptoms produced by one strain of a virus. Indeed, for many years now, plant breeders have been producing varieties of crop plants that die when infected with the common strains of prevalent varieties. This confers a practical control in the field, for infected individuals do not remain as sources of virus for their neighbours. The strains prevalent in a variety are those that do not kill it. In potatoes, however, the principal of breeding varieties hypersensitive to potato virus X is complicated by the many strains of this virus which affect different varieties differently, and by the occurrence of new strains that are less virulent and do not kill the variety.

J. S. K. BOYD: I apologize to Dr Bawden for my ignorance of the fact that a life cycle based on mutation from symbiotic to virulent has been recognized in plant viruses for many years. His general confirmation of my conclusions is gratifying.

THE NATURE OF PHAGE REPRODUCTION

ANDRÉ LWOFF

Pasteur Institute, Paris

WHAT IS REPRODUCTION?

Reproduction is the phenomenon by which an organized structure gives rise to identical organized structures. When a ciliate or a bacterium grows and divides, producing two organisms, they are supposed to have reproduced by binary fission. But, as pointed out by Bawden, binary fission can only be regarded as the last step in the process of reproduction of an organism. It is clear that binary fission cannot exist at the molecular level. And as certain specific molecules or particles, such as genes, are perpetuated, it is generally admitted that in some way they 'induce' the synthesis or organization of identical structures. From an operational point of view, it seems as though some specific molecules are endowed with genetical continuity, although, probably, the old structure never being present in the new one, provides only a 'cast'. Thus, at first sight, the reproduction of a molecule is something quite different from the reproduction of an organism. And as an organism is composed of molecules, this would mean that the nature of the reproduction of the parts is different from the nature of the reproduction of the sum. A disturbing conclusion.

WHAT IS NATURE OF REPRODUCTION?

An organism is essentially an organized structure, organization being the result of the spatial distribution of specific molecules or particles. Before division of an organism, the specific molecules are duplicated. Two daughter organisms are organized by virtue of the interplay of forces exerted by various particles. Fission separates two already organized structures. The problem of the nature of reproduction is the problem of the nature of the duplication of morphogenetical forces, that is to say of something which cannot divide because it is devoid of independent existence. Considered from this point of view, reproduction of a complex molecule and of an organism have something in common, namely, the organization of specific building blocks into specific structures or patterns.

It is nevertheless clear that a cell, or a non-cellular organism like a protist, is the minimum biological unit able to reproduce its own structure. And I am in perfect agreement with Bawden's statement according to which the multiplication of a virus is more comparable to the reproduction of cell constituents than to the reproduction of a cell or of an organism. Duplication of specific molecules or particles, including viruses, can only take place in a specific cellular environment, because, as the duplication of the cell itself, it is the result of the activity and the interplay of a great number of other specific molecules.

WHAT IS THE NATURE OF VIRUS REPRODUCTION?

When a virulent phage penetrates into a sensitive bacterium, it loses its infectivity. Infective particles appear after a dark period of some minutes. As 'phage' has not been seen with the electron microscope during the dark period, we have no precise data on the changes it undergoes. Analysis with U.V. and X-rays and studies on recombination have given some information on the subject. The fact that phage particles are remarkably uniform in size indicates that phage does not multiply by binary fission. From the fact that infectivity is lost after penetration, we may draw the same conclusion: phage is not reproduced as such. And the hypothesis that, when phage is reproduced, it has to develop from a non-phage structure seems plausible.

This conclusion is in agreement with general concepts concerning reproduction. Reproduction is the result of development. At each generation, the organism has to be built up anew. A sea-urchin has to be produced from an egg, a phage from a 'non-phage'. The expression 'reproduction by binary fission' is misleading. A 'non-cellular' organism is unable to divide, but has to organize two daughter organisms in the parental frame. As stated before, cleavage or fission, separates two already formed organisms. Living organisms, specific particles, structures, molecules, are never multiplied by fission, but produced only by organization of more or less complex building blocks. Phage, as probably other viruses, conform to this general law and every one certainly deplores the fact that the intimate 'nature' of the organizational process is unknown. Alternations of infectivity and non-infectivity are known not only in phage and other viruses but also in many organisms, and especially in protozoa such as malarial parasites, trypanosomes, ciliates, etc. . . .

Plasmodium malariae may multiply by schizogony in its mammalian host or in the mosquito by sporogony (associated with sexuality). Such a dual situation exists in bacteriophage which may be reproduced in sensitive bacteria by a process associated with recombination yielding infectious particles, and, in lysogenic bacteria as non-infectious particles.

Therefore phage reproduction, *sensu latissimo*, may take place by two processes: a process culminating in production of phage, a process in which phage is not produced, but reproduced as a potential phage.

This phenomenon, and the conditions necessary for phage production from a non-phage structure, which is phage reproduction, will be the main object of our attention.

INTRODUCTION OF THE PROPHAGE

Lysogenic bacteria perpetuate hereditarily the property to produce phage. A lysogenic bacterium survives only if it does not produce phage. Phage is not formed nor liberated during normal growth. The production of phage is not compatible with the survival of the microbe since it is killed when phage is formed. Phage is never 'secreted' but liberated by bacterial lysis.

Let us consider a non-lysogenic sensitive clone and a 'temperate' phage (temperate phages are phages able to form lysogenic systems; they are opposed to virulent phages which are devoid of this property). The sensitive clone is non-homogeneous. All the germs are receptive, able to adsorb phage. The majority are sensitive; they allow the development of phage which lyses its host. A small percentage are receptive but 'immune'. They are not lysed, continue to multiply and in many cases give rise to lysogenic clones. The phage produced by these 'lysogenized' clones is generally identical to the original phage. Something specific is therefore reproduced in lysogenic bacteria.

In 1929, Burnet and McKie, studying a lysogenic strain of *Salmonella enteritidis* discovered that the bacteria did not contain bacteriophages. They concluded that, in lysogenic bacteria, the phage must multiply in the form of a non-infectious model. The phenomenon was rediscovered independently in 1936 by E. and E. Wollman, confirmed the same year by Gratia (1936*a*), and later on by Lwoff & Gutmann (1950), who proposed the term *prophage* for the non-infectious form of the bacteriophage.

A lysogenic bacterium survives only if it does not produce phage. Perpetuation of the prophage is compatible with survival. But when prophage develops into phage, the bacterium is lysed. Before studying the reproduction of phage we have to consider the phenomena which occur during the production of phage from prophage.

PHYSIOLOGY OF IRRADIATED BACTERIA

Let us consider a population of *Bacillus megatherium* 899(1) growing exponentially. A small number of bacteria produce phage spontaneously. The proportion of the phage-producing bacteria varies with the nature of the medium, and the importance of the population. It will be seen later why. It represents roughly 1 per 1,000 of the population. Let us neglect this small fraction. If the culture is irradiated with U.V. rays (Lwoff, Siminovitch & Kjeldgaard, 1950*a*, *b*), almost all the bacteria will, after 45 to 60 minutes, lyse and liberate approximately 100 phage particles. Irradiation has induced phage formation with consequent bacterial lysis. What happens during the latent period which separates the inducing shock from the lysis?

In a complex medium (yeast extract+casein hydrolysate) an induced population of *B. megatherium* 899(1) continues to grow during the latent period. The growth rate decreases continuously. The first free phages are liberated around 43 minutes at 37°. If bacteria are lysed with lysozyme at regular intervals after induction, one sees that the first infectious particles appear around the 34th minute (Lwoff *et al.*, 1950*a*, *b*). These figures are of course not absolute. The length of the latent period is increased by a decrease in temperature, by an increase of the U.V. dose (Siminovitch & Rapkine, 1952).

Bacterial growth continues during the latent period. This 'residual growth' had been found in all inducible bacteria which have been examined: *Staphylococcus aureus*, *Pseudomonas pyocyanea* (Jacob, 1951*a*) and *Escherichia coli* K-12 (Weigle & Delbrück, 1951). It is possible that it exists also in non-inducible lysogenic systems in which owing to the small proportion of phage-producing bacteria the phenomenon will be difficult to recognize. Residual growth is nevertheless not compulsory for phage maturation. It may be suppressed in *pyocyanea* by submitting the bacteria to a low and constant carbon diet (Jacob, 1951*a*). Then phage develops normally, but no bacterial growth takes place. If the regimen, the amount of nutrient per unit time is too small, it becomes the limiting factor for the phage, the latent period is increased and phage takes everything for itself (Jacob, 1951*a*). A *pyocyanea* needs 2×10^{10} molecules of glucose in order to divide. In order to build up 100 phages, only 4×10^{9} molecules of glucose are needed. Things happen as if the developing phage would always consume what it needs, the bacteria benefiting only from the surplus, the residue of phage nutrition.

Bacterial syntheses continue during the latent period. Ribonucleic acid (RNA) and respiratory enzymes are synthesized. Both curves of RNA and of respiration are parallel to the bacterial growth curve (Siminovitch & Rapkine, 1951). Moreover, after induction, formation of adaptive enzymes is possible. In induced *pyocyanea* and *E. coli* K-12, the possibility to synthesize an adaptive enzyme is unimpaired during 10 minutes and then decreases (Jacob, 1951*b*; Siminovitch & Jacob, 1952).

The situation in lysogenic bacteria is therefore different from the situation in ordinary systems which have been studied: when a virulent phage infects a sensitive bacterium bacterial growth, synthesis of RNA (Cohen, 1946) and of respiratory enzymes (Cohen & Anderson, 1946) are completely inhibited and synthesis of adaptive enzymes is impossible[1] (Monod & Wollman, 1947). On the contrary, evolution of desoxyribonucleic acid (DNA) is parallel in sensitive bacteria infected with a virulent phage and in induced lysogenic bacteria: absence of DNA synthesis during the first half of the latent period, rapid increase during the second half (Siminovitch & Rapkine, 1951). Considering the fact that induced lysogenic systems are able to perform many syntheses, the absence of DNA synthesis during the first half of the latent period takes its full signification.

Physical inducers

The minimum dose of U.V. necessary to induce 100% of the bacteria of *B. megatherium* 899(1) (efficient dose) varies with the medium in which the bacteria have grown (Lwoff, 1951). It is 125 ergs/mm.^{2} for bacteria grown in yeast extracts, or 3000 ergs/mm.^{2} for bacteria grown in synthetic medium. The 'aptitude', the property of bacteria to react by phage formation to the U.V. shock, thus varies with the nutritional state of bacteria. The situation is complicated by the fact that aptitude may express itself or not according to the medium in which the bacteria are placed after the shock. For example 'synthetic' bacteria, submitted to an irradiation of 1250 ergs/mm.^{2} will continue to grow normally in the synthetic medium. They will produce phage and lyse if subcultured in a mixture of various amino acids (none of them seeming specific) and diluted broth.

Moreover, if bacteria grown in the presence of yeast extract are irradiated and left in yeast extract, they produce phage and lyse. But placed in broth after irradiation, they grow normally. This is a chemo-restoration. A mere change of medium may thus allow the expression of aptitude or may suppress the effect of U.V. irradiation (Lwoff, 1951). The situation appears complex.

X-rays have the same inducing effect as U.V. The effect of both rays is suppressed by visible light.

Chemical inducers

When studying the kinetics of phage formation during bacterial growth, it was observed by Lwoff, Siminovitch & Kjeldgaard (1950):

---

[1] One most not forget that, for the conveniency of phage study, highly virulent phages and highly sensitive bacteria were selected. It remains therefore possible that residual growth will be found also in some ordinary systems a very small number of which have been adequately studied.

1. that phage production, that is to say the ratio phage/bacteria, was constant during the exponential growth phase;
2. that when the growth rate was limited by oxygen tension, important phage production was observed. As much as 30% of the bacteria sometimes lysed and liberated phage.

This type of phenomenon could not be reproduced at will, but was observed from time to time. It nevertheless suggested that oxydoreduction phenomena could play a role in induction. It was then found (Lwoff & Siminovitch, 1951*a*) that reducers have an inducing effect: if a culture growing in yeast extract is supplemented with reducers such as thiomalate, thioglycolate, reduced glutathione or ascorbic acid, they show residual growth, lyse and liberate phage.

Induction by reducers depends on the nature of the medium and we shall therefore distinguish 'active' and inactive media, according as reducers exert or not an inducing effect.

Some samples of baker's yeast extract are active, some are not. Peptones, broth, synthetic media are inactive. Bottom yeast extracts may be by themselves active without any addition of reducers. If an active yeast extract is treated by 'Norite A' at pH 3·5, it becomes inactive. One—or many—substances have been removed which are necessary for the inducing activity of reducers.

These substances have recently been identified, and are referred to in the Appendix.

INDUCIBLE AND NON-INDUCIBLE SYSTEMS

U.V.-rays induce phage development in many lysogenic species. But all systems are not inducible. For example, *B. megatherium* 899(1) is inducible, whereas 17(2) is not (Ionesco, 1951). *E. coli* K-12 is inducible (Weigle & Delbrück, 1951) whereas *E. coli* Lisbonne (Bertani, 1951) is not. Is inductivity due to the properties of the bacteria or of the prophages? To answer this question, the phages 1 and 2 of *B. megatherium* have been studied by Ionesco (1951). Each of them has been transmitted to strain M and phage 1 to strain 17(2). The results are summarized in Table I. It is seen that 899(1), M(1) and 17(2) (1) are inducible, yielding phage 1 after induction. On the contrary, 17(2) and M(2) are not inducible. The system M(1) is inducible, the system M(2) not inducible. Prophage 1 is inducible in the strains 899, 17 and M whereas in the strain 17 and M, prophage 2 is not inducible. This means that inductivity depends on the properties of prophage. But it does not mean that inducing agents act on the prophage itself. It could well be

Table 1

Lysogenic strains	The produced phage is active on:	Phage produced spontaneously or after induction in inducible systems	Inductibility
899(1)	M, M(2), 17(2)	1	+
M(1)	M, M(2), 17(2)	1	+
17(2) (1)	M, M(2), 17(2)	1	+
17(2)	M	2	0
M(2)	M	2	0

From Hèléne Ionesco, 1951.

that the development of each phage is controlled by specific factors produced by bacteria, which production could, or could not, be induced by U.V.

It is clear that, in lysogenic systems we are faced with at least two types of prophages: (*a*) those whose development takes place spontaneously and which have not been induced either because we have not found the 'activator', or because they are really not inducible; (*b*) those which are inducible.

If we admit that the development of each prophage is controlled by specific factors, we may conceive the following hypotheses:

1. in inducible systems, the 'activator' is made available or produced by the bacteria as a consequence of the metabolic change induced by the shock;
2. in a non-inducible system the spontaneous production or availability of the activator is controlled by a bacterial gene mutation.

WHAT IS PROPHAGE?

Prophage may be defined as the form in which the potentiality to produce phage is perpetuated in lysogenic bacteria. Before discussing the nature of prophage, it is useful to consider briefly the problem of perpetuation of potential properties.

Perpetuation of potential properties

It is well known that some genes exert their action only in given circumstances: at a certain phase of the life cycle of the organism, at certain temperatures, when certain substances are present, etc. Thus genes appear as the stronghold of potential features. It is known also, that, in many cases, genes can act only in the presence of certain cytoplasmic particles.

The synthesis of chlorophyll, which is controlled by many genes, can take place only if the cytoplasm contains plastids; synthesis of cytochrome oxydase and of succinodehydrogenase in yeast, only if a certain plasmagene is present (see Ephrussi, 1951). Plastids, and Ephrussi's plasmagene of yeast, are endowed with genetical continuity. Here then, the expression of a property, specific synthesis, depends on the presence of two specific particles, the gene and a 'plasmagene' both of which are endowed with genetical continuity.

Now, what is prophage? As phage itself is essentially a desoxyribonucleoprotein, if we are to assume a material continuity from phage to prophage and from prophage to phage, four hypotheses may be considered. Prophage is perpetuated: (1) as a nucleoprotein, (2) in two separate parts, a protein and a desoxyribonucleic acid (DNA), (3) only as DNA, (4) only as protein.

The hypothesis that duplication of a specific DNA plays an essentia role in the perpetuation of prophage is in accordance with prevailing thoughts. It has been stated by Lederberg (1950) that lysogenicity in *E. coli* K-12 is segregated as the genes during crosses, but experimenta data supporting this conclusion have not yet been published. If the results are conclusive, this would mean that, in *E. coli* K-12, lysogeny is controlled by a gene-like structure. We know that the biosynthesis of many specific proteins is gene controlled. The chromosome located structure could be a gene controlling an enzyme whose activity is necessary for phage development. Things would of course be more interesting if it were the specific DNA of the phage itself.

The study of disappearance of lysogeny should provide data on the nature of prophage. This phenomenon was observed apparently for the first time by Gratia (1936*b*) in *B. megatherium*. Owing to Clark and Cowles' observations (in Lwoff & Siminovitch, 1951*b*) confirmed by Lwoff & Siminovitch (1951*b*), it is now possible to cure at will *B. megatherium* 899 from its lysogeny by serial transfers in synthetic media As observed by Gratia, cured, non-lysogenic *B. megatherium* are sensitive to the phage produced originally. This sensitivity could easily be explained in terms of selection. If a sensitive strain is composed of a majority of sensitive and of a few immune bacteria, it appears to be because 'sensitives' have a selective advantage over 'immune'. In a lysogenic strain only immune bacteria, in which prophage is normally unable to develop into phage, can survive. Every mutation from immunity towards sensitivity would produce the development of prophage into phage and bacterial lysis. But if the prophage is lost, the sensitive mutants may survive in a medium in which phage concentration is low and adsorption poor, and will be selected if they have a selective advantage. Coming back to the loss of lysogenicity, many hypotheses may be considered. But it seems difficult to escape the conclusion that curing is the result of the loss of a specific particle endowed with genetical continuity, a process of which many examples are known. Prophage may be a plasmagene. If the relative speed of its multiplication is lowered, microbial reproduction will finally produce prophageless bacteria. It is possible also to conceive of an accidental loss of a piece of bacterial chromosome containing the gene responsible for phage development.

Curing of lysogeny has been obtained only in *B. megatherium* and observed, accidentally, as far as we know, in *E. coli* K-12. This restricts the discussion. One fact remains: irreversibility of the loss of lysogenicity which probably means a loss of a specific particle endowed with genetical continuity. If the prophage is a particle, curing of lysogenicity means loss of the prophage. It will be seen later that in some strains of *B. megatherium* the development of prophage may be induced, but is abortive owing to a defect in the DNA synthesis. This favours the hypothesis of a dual nature, possibly protein and DNA, or cytoplasmic and nuclear, of the prophage.

Prophage as a vaccine

In a sensitive strain of *B. megatherium*, some 'resistant' bacteria are present which are not lysed by phage. These resistants, which give rise to lysogenic clones, are, by definition, immune. Let us admit that immunity is controlled by a spontaneous bacterial mutation, and exists before phage penetration.

In the absence of phage, sensitive bacteria are selected. In the presence of phage, sensitive bacteria are killed, and immune ones are selected. These are lysogenic. As has been seen, if prophage is lost, sensitive mutants are once more selected. The presence of prophage is therefore not responsible for immunity. But the presence of prophage is necessary in order that an immune bacterium should be selected. From an operational point of view, prophage is a vaccine, because in an environment where phage is found, only immune bacteria are able to survive, and because immune bacteria are selected only in the presence of phage. In the presence of phage, prophage is necessary in order to avoid epidemics. Of course, from time to time, prophage develops into phage and some bacteria are killed. This is the price the population has to pay for its maintenance. Individual 'sacrifices' are to be neglected when one considers the interest of the species.

APOVIRUSES

Prophage is a non-infectious particle, unit, or concept. And so far as we know, when it multiplies as prophage, it behaves as a normal particle and lacks two of the main features of a virus: infectivity and pathogenicity.

As prophage may develop into phage, either spontaneously or after induction, prophage is obviously a potential virus, the germ of a virus or the non-virus phase of a virus. This seems quite clear. But one has to remember here that the prophage 1 does not kill *B. megatherium* 899(1), that the multiplication of prophage 1 does not kill the host. The bacterium is killed only when prophage 1 develops into phage. This leads to a discussion of the cases in which bacteria are disturbed or killed by abnormal biosynthesis without any production of infective particles. Existence of intermediates between viruses and enzymes will thus be disclosed.

Let us cure *B. megatherium* 899(1) of its lysogenicity. The new strain 899(1d) (d for *deletum* = cured) does not lyse any more after U.V. treatment, does not produce phage, and is sensitive to phage 1. The cured strain 899(1d) is mixed with phage 1 and the survivors isolated. Most of them are lysogenic and most of these new lysogenic strains behave as 899(1). After irradiation they lyse, each lysed bacterium liberating on the average 100 phages. But one of the strains, 899(1d) 91(1) which will be designated simply by 91(1) exhibits a strange behaviour. It is lysogenic. In exponentially growing cultures, one finds 1 phage for 1,000 bacteria. If one bacterium liberates 100 phages, this means that one bacterium out of 10^5 has liberated phage spontaneously. After irradiation, 91(1) shows the classical picture: residual bacterial growth during the latent period and lysis. But no phages are liberated. One may then question whether lysis of 91(1) is bound to the presence of prophage or to a property of the bacteria. If 91(1) is cured, the new, non-lysogenic strain 91(1d) is not lysed any more after U.V. irradiation. It seems therefore established that

Table 2

	Lysogenic	Lysis after U.V.	Phage production after U.V.
899(1)	+	+	+
899(1d)	0	0	0
899(1d)(1)	+	+	+
899(1d)91(1) = 91(1)	+	+	0
91(1d)	0	0	0

lysis of 91(1) after irradiation is due to an abortive development of prophage (Lwoff & Siminovitch, 1951*b*).

Two questions immediately arise:

(*a*) *Is* 91(1) *producing a phage that we are unable to detect?* Electron microscopy allows a negative answer.

(*b*) *Why is development abortive?* Estimations of desoxyribonucleic acid during the latent period show: (1) that during the first half of this period there is, as in 899(1), no increase of DNA; (2) that during the second half of the latent period, the synthesis of DNA is very small if compared with the synthesis in induced 899(1) during the same period (Siminovitch, 1951).

From all this one may conclude: (1) that the abortive development of phage is due to a defect in DNA synthesis; (2) that U.V. dissociates two phenomena in the development of the prophage; (3) that the phenomenon (or phenomena) which takes place in irradiated 91(1) is accompanied by bacterial lysis.

An obvious hypothesis is, that in 91(1), U.V. light induces only the development of the protein part of the phage.

If we neglect the one bacterium in 10^5 which spontaneously produces bacteriophage, 91(1) will appear as a strain perpetuating a potential lethal factor. We know that the potential lethal factor is prophage, that we are able to induce only one part of its development, and that bacteria are killed without virus production. The 91(1) situation is a bridge between lethal action due to virus production and lethal or pathological action caused by the *production* of some proteins or enzymes.

E. coli ML, grown in a synthetic medium does not produce antibacterial substances. If irradiated with U.V., it continues to grow for 70 minutes and then lyses liberating substances which kill *E. coli* Bordet in a few seconds (Jacob, Siminovitch & Wollman, 1951). The synthesis of the active substance starts immediately after irradiation and the 'antibiotic' is liberated only by the lysis of the bacteria. The substance, so far as is known, and so far as the heterogeneous group of colicines is defined, may be labelled a colicine.

A similar situation has been described in *P. pyocyanea* 10. One of the strains, irradiated, synthesizes a substance, pyocine, which is liberated by lysis and is bactericidal for a strain of the same species (Jacob *et al.*, 1951).

In the colicinogenic *E. coli* ML and in the pyocinogenic *P. pyocyanea* 10, irradiation induces the synthesis of the bactericidal substance, just as it induces the development of prophage into phage in some lysogenic bacteria. *E. coli* ML and *P. pyocyanea* 10 are not sensitive to the colicine and the pyocine they respectively produce, just as lysogenic bacteria are not sensitive to their own phage. Synthesis of colicine and of pyocine, just as development of prophage into phage, is accompanied by the death of the 'producer' (bacteria producing the substance or particle). Colicine and pyocine, like phage, are liberated by lysis of the producer. *E. coli* ML, *P. pyocyanea* 10 perpetuate hereditarily the property to produce colicine or pyocine, just as lysogenic bacteria perpetuate hereditarily the property to produce bacteriophage. This is an interesting situation because it is possible to induce at will the expression of a potential lethal property, which is the synthesis of a bactericidal substance, perhaps of protein nature.

Table 3

Development of:	Effect of development *on the producer*	Effect of the synthesized substance or particle on other bacteria	The produced particle is (+) or is not (0) reproduced by the host
β-galactosidase	0	0	0
Protein Pz in galactose (−) bacteria	growth inhibition	0	0
Colicine and pyocine	death	death	0
Prophage (abortive)	death	0	0
Prophage into phage	death	death	+
The transforming principles	0	new syntheses	+

Another example is known of an induced biosynthesis of a specific protein which appears to be 'pathogenic' without being lethal. One particular mutant of *E. coli* ML is known which responds to galactose by a greatly increased synthesis of a protein (Pz) normally present in the cells. This effect is accompanied by a very marked inhibition of growth rate. Normal *E. coli* is *not* inhibited by galactose, and responds to it not by increased synthesis of Pz, but by synthesizing β-galactosidase, the protein of which is akin to, albeit distinct from the Pz protein (Cohen, unpublished).

The increased biosynthesis of protein Pz produces a disturbance of the growth, a typical disease of development due to malnutrition. Here is a beautiful example of a pathological biosynthesis, or better of a disease-producing, that is to say, a pathogenic biosynthesis.

All these facts are summarized in Table 3 to which data on the transforming principles have been added. With the exception of these transforming principles, which are probably genes or pieces of chromosomes, the substances or particles which have been considered are produced only as the consequence of the action of external factors or inducers. No phage, no colicine, no pyocine, are present in 'normal' bacteria. They are not reproduced as such, but the bacteria maintain hereditarily the power to synthesize them. It is interesting to note that the biosynthesis of protein Pz may bring about a disturbance of bacterial growth under special circumstances. And it is highly suggestive that the induced development of the other particles is lethal. The development of prophage into phage is lethal, whether abortive or not; the biosynthesis of colicine or pyocine is lethal.

The mechanisms of some pathological or lethal actions are thus to be found in pathogen biosyntheses, some of them corresponding to a protein, some others to a part of a virus. Viruses are not as isolated entities as they appeared and may thus be considered as the infectious members of a great family.

WHAT IS A VIRUS?

Time has come, perhaps, to give a definition of the subject of our attention. Phage may be considered as a model of a typical virus. When considering Table IV it is seen that each of the properties of phage may be found in one or more other types of particles. But virulent phage considered in its relation with a sensitive bacteria is at the same time pathogenic, able to penetrate a new host and to be reproduced. When present together these properties are characteristic of what may be called *true viruses.* It is possible to add that phage may reproduce only in a specific host. All

Table 4

	Power to be adsorbed or to penetrate in a new host	Reproduction in the receptive host	Pathogen or lethal for the receptive host	Perpetuated hereditarily as such	Development:		
					without effect	pathogen	lethal
Transforming principles	+	+	0	+	+	0	0
Protein Pz (in galactose- bacteria)	0	—	—	0	0	+	0
Colicine and pyocine	+	0	+	0	0	+	+
Prophage (in immune bacteria)	0	—	—	+	+	0	0[1]
Abortive phage	0	—	—	0	0	+	+[2]
Virulent phage	+	+	+	0	0	+	+

[1] Multiplication is not lethal. Development into prophage is lethal.
[2] Abortive development is lethal.

these features are shared by many obligate parasites which are not viruses, as for example, some parasitic protozoa.

In all cells, in all bacteria, protozoa or fungi, a nucleus and protoplasm may be clearly recognized. In phage, nucleic acid and protein seem, so far as we know, combined into one macrostructure. A specific nucleoprotein which kills the cell and which multiplies may only be a virus. The immediate objection of course is that if some viruses may perhaps be only a nucleoprotein, some others possess a tail, a membrane, and other differentiated organelles. This would not be so important if the nucleoprotein would be the only part endowed with genetical continuity, that is to say if the various organelles were the result of the 'development' of the nucleoprotein, of its organizing power. Thus, neglecting the secondary features, a *true virus* could be defined as *an infectious and pathogenic nucleoprotein* (or *pathogenic molecule* would be less compromising). But properties of viruses vary with the phase of the life cycle, with the nature of the host, and may be temporarily or permanently lost. It is felt that phylogenetical and ontogenetical features have to be included in a general definition of viruses; a definition which would *exclude* transforming principles, pathological proteins, such as colicine or pyocine and *include* latent or masked viruses or proviruses. It would be a Platonist concept, the lower limit of a virus: a molecule endowed with genetical continuity and able to behave as a virus or to develop a structure behaving as a virus. *The theoretical virus would be defined as a specific reproducible nucleoprotein or molecule which was, is, or may become infectious and pathogenic.*

If, between small bacteria and this noble, ideal being, intermediary steps do exist, it is no longer possible to play with a definition. Therefore, preference was given to a concept of a virus rather than to viruses in general. I am nevertheless perfectly conscious of the fatal consequences of this definition. But it was felt impossible to accept discussing the nature of virus reproduction without giving some sort of definition not of what viruses are, but at least of what they should be.

REFERENCES

BAWDEN, F. C. (1939). *Plant viruses and virus diseases*, Leiden.

BERTANI, G. (1951). Studies on lysogenesis. I. The mode of phage liberation by lysogenic *Escherichia coli*. *J. Bact.*, **62**, 293.

BURNET, F. M. & MCKIE, M. (1929). Observations on a permanently lysogenic strain of *B. enteritidis gaertner*. *Aust. J. Exp. Biol. Med. Sci.*, **6**, 277.

COHEN, S. S. (1946). The synthesis of bacterial viruses in infected cells. *Cold Spring Harbor Symp.*, **12**, 35.

COHEN, S. S. & ANDERSON, T. F. (1946). Chemical studies on host-virus interactions. I. The effect of bacteriophage adsorption on the multiplication of its host, *Escherichia coli B*. *J. Exp. Med.*, **84**, 511.

Gratia, A. (1936*a*). Des relations numériques entre bactéries lysogènes et particules de bactériophage. *C. R. Soc. Biol.*, **122,** 812.

Gratia, A. (1936*b*). Analyse et synthèse du pouvoir lysogène chez le *Bacillus megatherium. C. R. Soc. Biol.*, **123,** 506.

Jacob, F. (1950). Induction de la lyse et de la production de bactériophages chez un Pseudomonas pyocyanea lysogène. *C. R. Acad. Sci.*, **231,** 1585.

Jacob, F. (1951*a*). Suppression expérimentale de la croissance des bactéries au cours du développement du bactériophage chez *Pseudomonas pyocyanea. C. R. Acad. Sci.*, **232,** 1605.

Jacob, F. (1951*b*). Adaptation enzymatique pendant le développement du bactériophage chez *Pseudomonas pyocyanea. C. R. Acad. Sci.*, **232,** 1780.

Jacob, F., Siminovitch, L. & Wollman, E. (1951). Induction de la production d'une colicine par le rayonnement ultraviolet. *C.R. Acad. Sci.*, **233,** 1500.

Lederberg, E. M. (1950). Lysogenicity of *Escherichia coli* strain K-12. *Microb. Genet. Bull.*, No. 1, p. 5.

Lwoff, A. (1951). Conditions de l'efficacité inductrice du rayonnement ultraviolet chez une bactérie lysogène. *Ann. Inst. Pasteur*, **81,** 370.

Lwoff, A. (1952). Rôle des cations bivalents dans l'induction du développement du prophage par les agents réducteurs. *C. R. Acad. Sci.* (in press).

Lwoff, A. & Gutmann, A. (1950). Recherches sur un *Bacillus megatherium* lysogène. *Ann. Inst. Pasteur*, **78,** 711.

Lwoff, A. & Siminovitch, L. (1951*a*). Induction par des substances réductrices de la production de bactériophages chez une bactérie lysogène. *C. R. Acad. Sci.*, **232,** 1146.

Lwoff, A. & Siminovitch, L. (1951*b*). Induction de la lyse d'une bactérie lysogène sans production de bactériophage. *C. R. Acad. Sci.*, **233,** 1397.

Lwoff, A., Siminovitch, L. & Kjeldgaard, N. (1950*a*) . Induction de la lyse bactériophagique de la totalité d'une population microbienne lysogène. *C. R. Acad. Sci.*, **231,** 190.

Lwoff, A., Siminovitch, L. & Kjeldgaard, N. (1950*b*). Induction de la production de bactériophages chez une bactérie lysogène. *Ann. Inst. Pasteur*, **79,** 815.

Monod, J. & Wollman, E. (1947). Inhibition de la croissance et de l'adaptation enzymatique chez les bactéries infectées par un bactériophage. *Ann. Inst. Pasteur*, **73,** 937.

Siminovitch, L. (1951). Relation entre le développement abortif du prophage chez *Bacillus megatherium* 91 (1) et la synthèse de l'acide désoxyribonuclégique. *C. R. Acad. Sci.*, **233.**

Siminovitch, L. & Jacob, F. (1952). Synthèse adaptative de la β-galactosidase chez *E. coli* K–12 induit. *Ann. Inst. Pasteur*, (in press).

Siminovitch, L. & Rapkine, S. (1951). Modifications biochimiques au cours du développement des bactériophages chez une bactérie lysogène. *C. R. Acad. Sci.*, **232,** 1603 and *Ann. Inst. Pasteur* (in press).

Weigle, J. J. & Delbrück, M. (1951). Mutual exclusion between an infecting phage and a carried phage. *J. Bact.*, **62,** 301.

Wollman, E. & Wollman, E. (1936). Régénération des bactériophages chez le *B. megatherium* lysogène. *C. R. Soc. Biol.*, **122,** 190.

APPENDIX 1: FACTORS CONTROLLING DEVELOPMENT OF PROPHAGE INTO PHAGE

> '*Hence it may be profitable to think of cells and tissues of all kinds as areas in which traces of metallic ions are competed for by the various complex-forming agents present*' (Albert, 1950).

It has been stated that induction by 'reducers' takes place only in certain types of yeast extracts, not in broth nor in solutions of 'peptones'. Two types of media, 'active' and 'inactive', have been distinguished, according to whether they do, or do not, allow the inducing effect of reducing agents. And it was concluded that a substance, present in active media, is necessary for the inducing effect of reducers on *B. megatherium* 899.

In an active yeast extract, induction has been obtained by oxine (8-hydroxyquinoline) M/85,000 (Lwoff, 1952). The well-known bactericidal action of this substance is due to a displacement of Co^{++} which allows an acute lethal action of Fe^{++} and Cu^{++} (Rubbo, Albert & Gibson, 1950). Is the inducing effect of oxine bound to such a cationic permutation?

Treatment with oxine inactivates active yeast extracts. Activity is restored by Cu^{++} 10^{-5}M, which is not replacable by Fe^{++}, Mg^{++}, Ca^{++}, Zn^{++}, Mn^{++}, Co^{++}. Cobalt has an antagonistic effect: it prevents phage development in the presence of Cu^{++} and thioglycollic acid (Lwoff, 1952).

Inactive media allow bacterial growth and phage production after U.V. shock. They contain therefore all the elements necessary for bacterial growth and for phage development. It is only when induction is being effected by reducing agents that an excess of Copper becomes necessary, because the reducing agents themselves exercise metal binding properties.

Thus, induction appears to be the result of a cationic permutation: replacement of Co^{++} or of another 'protective' cation by Cu^{++}, either at the level of the prophage itself, or at the level of an enzyme controlling the development of prophage into phage.

The absence of phage development in lysogenic bacteria, condition of the very existence of lysogenic clones, is controlled by an equilibrium depending on the absolute and relative affinities of bacterial constituents for cations, and of the absolute and relative proportion of cations in the bacteria.

If it is admitted that the same specific effect, namely the development of prophage into phage, must depend on the same specific cause, it follows that all inducing agents or substances act in modifying the cationic equilibrium and allowing the right cation to reach the right place.

It was found with Kenneth Huybers (unpublished) that the inducing effect of U.V. shock, also depends on the cationic balance of the bacteria.

Thus, it does appear that the maintenance of the prophagic non-pathogenic, phase of the phage in *B. megatherium* is bound to a certain type of cationic balance. The pathological development of prophage into phage is bound to a disturbance of this balance.

APPENDIX 2: REPLICATION

(added 17 April, 1952)

Many hypotheses have been proposed for the mechanism of the duplication of genes or of other structures endowed with genetic continuity. L. T. Troland (1914) (quoted from Muller, 1947) considered that 'raw material'—we would say building blocks—become attached to like parts of the pre-existing genes, so that an identical structure be formed. Replication seems here the result of a *face-to-face* attachment.

To account for this face-to-face attachment, Jordan (1940) has invoked the quantum-resonance phenomenon. This concept has been criticized by Pauling and Delbrück (1940) who concluded from their calculations that the resonance interaction could not cause a specific attraction between like molecules, and therefore could not be effective in bringing about 'autocatalytic reactions'.

Pauling and Delbrück pointed out that maximum stability is achieved by bringing the molecules in contact in such a way that positively charged groups are brought near to negatively charged groups and reached the conclusion that *complementariness* should be given primary consideration in the discussion of the specific attraction between molecules and their synthesis.

Complementariness is also a part of Delbrück's concept (1941) according to which specific precursors, such as amino acids, are attracted and attached by the homologous structures of the specific molecule by means of a resonance occurring at the site of the peptide links. This implies according to Delbrück a *back-to-face* attachment. Thus the back of each molecule would have to correspond to the face of the other. As the two molecules are identical this implies that the back of each molecule involved is, in one way or another, complementary to its front. It is not known whether substances with such properties are found among the constituents of living organisms.

Thus, according to Pauling and Delbrück's, as well as to Delbrück's concept only complementary structures could become attached. However, as pointed out by Muller (1947), during the synapsis, the like parts of chromosomes become attached to one another two by two. Muller suggests, a view accepted by Delbrück, that the same principle is acting in gene synthesis and in chromosome synapsis. This, according to Muller would indicate a *face-to-face* attachment, a conclusion which is difficult to avoid. Two different phenomena, according to Fabergé (1942), have to be considered: (*a*) the specific attraction of chromosomes at long distance which occur at the zygotene phase, (*b*) the attachment resulting in two-by-two pairing. In process (*a*) there is no saturation: if more than two homologues are present, they all come together. In process (*b*) there is a saturation when two homologues are paired. The nature of the two processes are therefore different, and process (*a*) attraction has been explained by Fabergé by the Buyot-Bjerkness effect. This phenomenon is not dealt with in this discussion which concerns especially *attachment*.

When two molecules of a peptide are attracted by virtue of direct electrostatic forces, complementary structures would come into contact. The imino group of one peptide bond would come in contact with the carbonyl group of the other and *vice versa*, the molecules being held by direct electrostatic attraction or through rearrangement of the electronic distribution by the formation of co-ordinate links or hydrogen bonds. This implies a contact between opposite groups which is liable to take place only when *complementary* structures come into contact.

Thus a *direct* face-to-face attachment does not seem to be possible. And yet, so far as we know, such an attachment takes place during synapsis. Moreover, face-to-face attachment not involving the intermediary intervention of a complementary molecule is the simplest way of replication. Let us then look for known types of liaisons which could account for the alleged phenomenon. Among them is the disulphide bridge which is effective in the formation of reduced glutathione by oxidation of two reduced glutathione molecules. But –SH groups are rare in proteins, and absent in nucleic acids which are, presumably, the replicating materials.

It is known that biological substances such as amino acids, pterins, riboflavin, purine bases, may become attached *front-to-front* when they chelate with a divalent ion, and that the energy involved in this reaction is small.

Proteins are known to bind metals, and it is by the intermediary of divalent ions such as Co^{++}, Mn^{++}, etc., that some peptidases bind their substrates. Nucleic acids also have a strong avidity for metals.

Let us consider a linear molecule, whether a protein or a nucleic acid. The reactive groups would have a tendency to bind divalent cations such as Cu^{++}, Co^{++}, Fe^{++}, Mn^{++}, which, being indispensable parts of the living organisms, are always present in a normal cell and which seem to have been neglected in the theories concerned with replication. The mono-organic-polymetallic compound would have a strong affinity for a like structure or for the building blocks of a like structure. (In order to form a structure equivalent to the replicating molecules, the building blocks would have to be in a given position and state so that their adjacent groups could combine). The maximum stability would be reached when a symmetrical structure would be produced, composed of two identical organic molecules, face-to-face, in which homologous groups are bound by divalent cations. We are, of course, far from understanding the mechanism by which it is precisely the homologous 'blocks' which must be and are attracted and attached. The architecture and organization of the cell certainly plays an important part in the phenomenon. And it would be premature to try to explain such a complicated phenomenon in terms of our actual knowledge. The fact that very few or no data are available concerning the number of each specific molecular species involved, the number of ions, atoms and affinities of the ions for the micro- and macro-molecules under conditions prevailing in the cell, is a serious drawback. Nevertheless the proposed mechanism would account for synapsis and for replication. One might conceive also that the mono-organometallic compounds are able to bind not only *like parts* but *isosteric parts*. Thus a specific nucleic acid could attract not only purine and pyrimidine bases but, for example, amino acids or peptides and thereby organize or synthesize a specific protein.

It has to be noted that a face-to-face attachment between identical or like molecules can only take place between linear or two-dimensional structures. Globular structures, according to this concept, would be unable to produce replicas, at least by the mechanism here described.

Any theory concerned with replication and synapsis has to account, not only for *face-to-face* attachment but also for *separation* of the two identical structures. It also has to account for the fact that in the interval of two divisions a chromosome produces only one chromosome, a gene produces only one gene. As pointed out by Bawden and Pirie, it is more difficult to find out what stops syntheses than it is to find out what starts them. The involved mechanism has to be, in one way or another, controlled by the sequence of events occurring in the interval of two divisions. In the *face-to-back* attachment theory, the gene synthesis is able to proceed indefinitely, provided the building blocks are produced. In the *face-to-face* attachment theory, the synthesis is blocked when model and replica are combined into the alleged symmetrical structure. A new replication will be able to take place only when the replicated structure has been separated from the model, that is to say after 'division'. What is true for the cell is therefore true for the molecules.

Thus, some biological factors operating inside the cell have to be found which would control either attachment or separation. These factors would have to undergo a cycle and their appearance and disappearance would have to coincide with the life cycle of the cell. Their properties must be such that they could control attachment and replication on one hand, and separation of the parts of the complex on the other.

One substance undergoes cyclical changes connected with cell division: it is known, thanks to Louis Rapkine's classical work, that before division there is a considerable increase of soluble –SH groups. Such an increase would account for the rupture of disulphide bridges. Moreover, thiol groups are powerful metal binders.

Variations of the concentration of metal binding substances thus occur effectively in the cell in the interval of two divisions. These substances would, of course, compete for various ions with other cell constituents, and, among them, with molecules awaiting replication or with 'expecting complexes', formed by the replicated molecules and the chelated ions and awaiting separation. The result of the competition would depend on the concentration and relative affinity for cations of the organic molecules or complexes involved and on the concentration and nature of divalent cations.

It is possible to conceive that glutathione, whose role in cell chemistry in general and in cell division in particular is not clearly understood, could act either as a donor or as a receptor of divalent cations, that is to say as an agent of cationic movements.

Thus, owing to the interplay of the various cell components, a specific molecule would bind divalent cations, and attract corresponding building blocks, the result being replication; or the symmetrical structure would be dissociated by displacement of the binding divalent cations.

This picture was built up as a consequence of recent findings concerning the role of divalent cations and reducing substances in the development of prophage into phage. It is immediately obvious that the scheme does *not* apply to bacteriophage whose reproduction takes place without the intervention of any cell division, which, in the cationic scheme, is necessary for the separation of the model from the replicated structure. This is precisely what one would expect. If duplication of a virus obeyed the same laws as replication of normal cell constituents endowed with genetical continuity, the virus would be replicated only once in the interval of two divisions: viruses would not be viruses.

It may be supposed that some mechanism is common in the replication of normal structures and of viruses. What could be common would be attachment with the help of divalent cations. What could be different is the equilibrium between the cell constituents and complex 'model+cations+new structure'. The balance of affinity constants could be upset, either by increased or decreased affinity of one of the components of the system, leading to a separation of the newly organized structure from the model. The action of U.V. shock, or of reducing substance+copper, would be a modification of the affinities for divalent cations of either one or both members of the system.

APPENDIX 3

(added 13 May, 1952)

During the Oxford session, Dr J. Philpott suggested that peroxides produced as a consequence of U.V. irradiation could play a role in induction of prophage development, and that ascorbic acid acting as inducer could be a source of hydrogen peroxide. As a matter of fact, it is known that the antibacterial action of ascorbic acid is due to hydrogen peroxide production. In the course of a visit in Cambridge I had occasion to discuss, with Professor D. Keilin, the problem of the role of copper in the induction by thioglycolate. Professor Keilin told me that whenever he needed small quantities of hydrogen peroxide to be produced he used to mix a sulphydryl compound with copper.

The possible inducing effect of H_2O_2 had to be tested. It was discovered that H_2O_2 induces phage production in lysogenic *Bacillus megatherium* and *Pseudomonas pyocyanea* as well as colicine synthesis in *Escherichia coli* ML (A. Lwoff & F. Jacob, unpublished).

Induction has been obtained in *B. megatherium* with 8-hydroxyquinoline. The bactericidal effect of this substance which takes place in the presence of copper ions has been considered by Rubbo, Albert and Gibson as possibly due to an oxidative action of copper on some sulphhydryl compound.

Acknowledgment

The author's work has been supported by a research grant from the National Cancer Institute of the National Institutes of Health, U.S.A. Public Health Service.

REFERENCES

Albert, A. (1950). Quantitative studies of the avidity of naturally occurring substances for trace metals. I. Amino acids having only two ionizing groups. *Bioch. J.*, **47**, 531–538.

Delbrück, M. (1941). *Cold Spring Harbor Symp.*, **9**, 122.

Fabergé, A. C. (1942). *J. Genet.*, **43**, 121.

Jordan, P. (1940). *Z. ImmunForsch.*, **97**, 330.

Lwoff, A. (1952). Rôle des cations bivalents dans l'induction du développement du prophage par les agents réducteurs. *C. R. Acad. Sci.*, **234**, 366–368.

Lwoff, A. & Huybers, K. (unpublished).

Muller, H. J. (1947). *Proc. Roy. Soc.*, *B*. **134**, 1.

Pauling, L. & Delbrück, M. (1940). *Science*, **92**, 77.

Rubbo, S. D., Albert, A. & Gibson, M. I. (1950). The influence of chemical constitution on antibacterial activity. Part V: the antibacterial action of 8-hydroxyquinoline (oxine). *Brit. J. Exp. Path.*, **31**, 425–441.

DISCUSSION

M. WELSCH: In connexion with Dr Lwoff's report on lysogenic induction in *Bacillus megatherium*, I wish to report briefly a few observations made on lysogenic staphylococci in my laboratory, with the collaboration of Drs G. Cavallo, P. Cantelmo and J. Goffart-Roskam.

Lysogenic staphylococci can be induced by ultraviolet irradiation, addition of several SH-bearing compounds, and also by a very short heat-shock. Hydrogen peroxide or irradiated culture media are without effect.

Induction is, partially or completely, either prevented or cured by visible light illumination or incubation at 50° for half an hour.

Lysogenic staphylococci are sensitive to inducing agents not only when they are in the exponential phase of growth, but also when they are taken from cultures several days old and even from cultures starved by incubation in buffer.

Ultraviolet irradiation induces the Lwoff phenomenon in lysogenic staphylococci suspended in nutrient or non-nutrient fluid, provided that the cells are next brought into a nutrient medium and incubated.

After suitable irradiation, staphylococci can be stored in the ice-box for at least two days: when they are brought back to 37°, they display a typical Lwoff phenomenon.

If the growth of induced staphylococci is interrupted after various incubation periods by addition of antiseptics or antibiotics, lysis and liberation of phage occur as in the control, but only when growth has been allowed to take place for about half an hour or more. If, in similar experiments, the growth is interrupted by simple cooling the induced cultures in the ice-box, lysis occurs slowly in the cold, but without much liberation of phage, and only if the period of incubation following irradiation is at least as long as 30 minutes. After sub-optimal irradiation, this lysis in the cold can be the only visible effect of induction.

When induced bacteria are highly diluted and phage production is followed by plaque counts, phage liberation starts earlier and is more quickly completed than when estimates of phage production are based on turbidimetric methods.

With some strains, the turbidity of ultra-violet induced cultures steadily increases, whereas viable count shows a continuous drop and plaque count an increase of free phage.

I shall not attempt any theorizing about the facts reported here but I thought it was worth while bringing them to your attention in order to underline the general occurrence of Lwoff's phenomenon as well as the differences in behaviour of different species or strains.

J. D. WATSON: In connexion with Dr Lwoff's remarks on the role of cations during phage reproduction, I should like to mention the unpublished results of Hershey and Chase on the structure of bacteriophage T2. They find that the nucleic acid is located in the interior of the phage particle and is surrounded on the outside by the protein component. As the nucleic acid comprises 40% of the total virus mass, the radius of the nucleic acid core must be approximately 220A° and the thickness of the protein shell approximately 90A°. Since the diameter of a nucleic acid chain is unlikely to be greater than 22A° (so M. H. F. Wilkins tells me), the negative charges on the nucleic acid chain cannot be neutralized by the basic amino acids of the virus protein. It is thus probable that the nucleic acid component of the bacteriophage particle is in the form of a metallic salt. Unfortunately no analysis of the ionic contents of phage particles has been reported.

Dr Bergold tells me, however, that he has recently started a study of the ionic contents of a polyhedral virus and that he finds only Mg^{++} present in significant amounts. It thus seems possible that the DNA of the polyhedral viruses is also present as a metallic salt.

A. ALBERT: Dr Lwoff's manuscript states: 'It is only when induction is being effected by reducing agents that an excess of copper becomes necessary, because the reducing agents themselves exercise metal-binding properties.' In this connexion, I should think it would be interesting to use ascorbic acid as a reducing agent because it does not combine with metals to any extent: thus the experimental results should become easier to interpret because one variable would be removed.

I think it would also be interesting to include nickel in the tests. A few years ago, Mellor and Maley discovered the interesting fact that the majority of metal-binding substances have a definite order of preference for metals, viz:

$$Cu^{++} > Ni^{++} > Co^{++} = Zn^{++} > Fe^{++} > Mn^{++} > Mg^{++}.$$

Thus, of the divalent cations, copper is the most avidly bound. Now, Dr Lwoff has shown that Cu^{++} has a potentiating action on the development of prophage and Co^{++} is antagonistic. Is this a specific effect or merely a consequence of the order of these metals in the Mellor and Maley series? This could be decided if Ni^{++} were included in the tests.

L. F. HEWITT: Some recent experiments in my Institute emphasize the point that the mechanism of bacteriophage multiplication and the phenomenon of lysogenesis are not of merely academic interest but may be of importance in epidemiology and the evolution of bacterial variants. In seeking an explanation for the large number of different serological types of *C. diphtheriae* encountered in infections it was found that a high proportion of diphtheria strains were lysogenic. The diphtheria phages were found to be type-specific and in some cases the degree of specificity was higher than that seen in serological reactions such as agglutination. In the case of two phages active against a *mitis* strain experiments showed that some other strains of the same serological type were susceptible to the phages while others were completely resistant. The resistant strains were virulent and toxigenic while the susceptible strains were avirulent and non-toxigenic. Although indistinguishable serologically the strains were segregated by phage-susceptibility into virulent and avirulent subtypes. Furthermore the resistant mutants which grew out when six susceptible avirulent strains were sub-cultures in the presence of phage proved to be fully virulent and toxigenic. On the other hand with strains of some serological types phage-resistant mutants of greatly diminished virulence and toxigenicity were obtained. Recently Dr Freeman of Seattle has also observed that an avirulent strain became virulent after treatment with bacteriophage.

Thus phage constitutes a potent naturally-occurring agent capable of enhancing or diminishing the virulence of pathogenic bacteria and this phage-selection may account for the rise and fall in the gravity of epidemics which until now have been something of a mystery. Recently strains of *C. diphtheriae* have been sent to me isolated from fully immunized communities. These strains are avirulent and might be dismissed as of no significance but in the presence of the appropriate bacteriophage they might well become the source of virulent epidemic-producing agents among the non-immunized.

H. WILLIAMS SMITH: The induction of lysis in lysogenic bacterial cultures by means of chemical methods appears to have a fairly wide application. I have been able to bring about a great increase in the number of phage particles liberated by some lysogenic strains of *Salmonella* by means of sodium thioglycollate, glutathione, and nitrogen mustard, but not by ascorbic acid. For the particular broth in use, the optimum amount of sodium thioglycollate was in the region of 0·05%. When amounts larger than 0·2% of either sodium thioglycollate or of ascorbic acid were added to the medium, phage liberation either decreased enormously or ceased.

The results of adding sulphonamides to the medium were, perhaps, even more interesting. Sulphathiazole in amounts just sufficient to cause a slight decrease in the bacterial growth rate brought about a great increase in the liberation of phage particles by some lysogenic *Salmonella* cultures. This was not noted when small amounts of para-amino-benzoic acid were added to the medium suggesting that this increased liberation was related to the direct action of the sulphathiazole on the bacteria themselves. Although conclusions drawn from bacteriophage experiments may in certain cases reasonably be applied to animal virus infections, in other cases such correlations may be dangerous. It may well be that an attempt to correlate the results of the sulphathiazole experiments with the observation that the administration of sulphonamides in some animal virus infections may exacerbate clinical symptoms would be relegated to the latter category.

A. LWOFF: F. Jacob has recently shown (unpublished) that nitrogen mustard is an excellent inducer of phage development in *Bacillus megatherium*, *Pseudomonas pyocyanea* and *Escherichia coli* K–12. Nitrogen mustard also induces colicine formation in *Escherichia coli* ML. It is devoid of inducing effect on *Escherichia coli* Lisbonne which is not induced by U.V.

G. S. STENT: A mechanism of replication may be conceived which involves neither templates nor face-to-face or face-to-back attachments. Instead, a process of 'crossing-over' in which homologous pieces are exchanged between contiguous structures could be the mechanism by which small building blocks are assembled into large polymers of a given specificity.

Consider a mother molecule *ABCDEF* which is to be duplicated in an environment containing the necessary building blocks A', B', C', D', E', F'. The mother molecule splits into two fragments

$$\begin{array}{cccccc} A & B & C & & & \\ & & & D & E & F \end{array} \qquad (1)$$

and only that building block may fit on to the broken end which corresponds to the one to which the end had been joined before the break, i.e.

$$\begin{array}{cccccc} A & B & C & D' & & \\ & & C' & D & E & F \end{array} \qquad (2)$$

Duplication is thus under way, but the newly-joined building blocks at the ends of the fragments do not yet possess the correct 'twist' to ensure that only the right block could be joined next. By 'crossing-over' between the two fragments, i.e.

$$\begin{array}{cccccc} A & B & C' & D & & \\ & & C & D' & E & F \end{array} \qquad (3)$$

the newly-joined blocks C' and D' are placed into the interior of the molecule, and the homologous blocks of the mother molecule C and D possessing the right 'twist' are at the ends. Now only the correct blocks may be joined on, i.e.

$$\begin{array}{cccccc} A & B & C' & D & E' & \\ & B' & C & D' & E & F \end{array} \qquad (4)$$

Again, 'crossing-over'

$$\begin{array}{cccccc} A & B' & C & D' & E & \\ & B & C' & D & E' & F \end{array} \qquad (5)$$

and finally

$$\begin{array}{cccccc} A & B' & C & D' & E & F' \\ A' & B & C' & D & E' & F \end{array} \qquad (6)$$

i.e. duplication is complete.

The operational distinction of this conception of replication is that the atoms of the mother molecule are necessarily distributed evenly over the two molecules resulting from duplication. Under template and attachment theories, the atoms of the mother molecule would not appear in the daughter structures.

A. LWOFF: Dr Stent's theory is very attractive. Yet it does not seem to account for the face-to-face attachment of chromosomes. Moreover it is difficult to visualize the nature of the factors responsible for the splitting of the mother structure. Nevertheless I do not think that Dr Stent's scheme is more devoid of any positive evidence than any other theory of replication. We are now faced with half a dozen such theories and it may be hoped that one or the other may soon serve as a working hypothesis.

THE MULTIPLICATION OF BACTERIAL VIRUSES STUDIED BY RADIOBIOLOGICAL METHODS

RAYMOND LATARJET

Radium Institute, Paris

This paper only endeavours to describe how radiobiological methods can be successfully used as a tool for investigating the multiplication of certain viruses, and to define the contribution of these methods in the study of a few bacteriophages which differ in size and in organization.

As early as 1947, these methods disclosed that the processes of virus growth are far more complex than it was originally believed, and that they follow particular pathways thus far unknown within the realm of living organisms. Of course, the numerous viruses, so different in size, in structure, in behaviour, and in host affinities, may multiply within their respective hosts through numerous processes. Nevertheless, any new finding concerning one of them can be of suggestive and valuable assistance in the study of others.

Ionizing and ultraviolet radiations inactivate all virus particles, either free or intracellular, thanks to very localized absorption of radiant energy, followed by chemical reactions. As these minute and discontinuous events start at the molecular level, these radiations behave, for this inactivation, as a micro-gun whose separating power is 10 to 100 times as high as that of the electron microscope. Radiations easily 'see' the virus particles, and, once we know the nature of the absorbing molecules, we may say that they 'see' these molecules. The prerequisite to these methods is that one can determine the rate of inactivation produced by any dose of radiation. For this purpose the most precise way is to number one by one the infective centres (either free particles or infected cells), before and after irradiation. These methods have therefore been applied so far only to bacterial viruses. But, as irradiation is tantamount to a dilution, they could be successfully applied—although with greater experimental difficulties—to animal and plant viruses. Their main advantage lies in the fact that radiations perceive the virus in its actual situation within the host, without any preliminary and possibly disturbing treatment, such as fixation, colouration, breakage, or lysis of the host cell. On the other hand, they can be worked out only on rather large populations of infected cells, in such a way that their results only express the average picture of what actually happens in many individual cells.

BASIS OF THE RADIOBIOLOGICAL METHODS

(*a*) *Inactivation of extracellular viruses*

Ionizing and UV-radiations inactivate free viruses with high efficiency. The photochemical patterns of this phenomenon are still unknown. If one irradiates a virus suspension with various doses of a given radiation, one obtains the 'survival curve' by plotting the ratio of non-inactivated particles against the dose. In the case of ionizing radiations, this curve is always exponential, as was first shown by Wollman & Lacassagne (1940). For UV it is generally exponential (Latarjet & Wahl, 1945), although some large viruses give a more complex curve of the sigmoid type (Latarjet & Morenne, 1951). An exponential relationship between survival and dose indicates a one-hit phenomenon: inactivation of a particle is the end result of a chain of events initiated by the absorption of a single quantum of radiant energy. Such an *efficient* absorption is accompanied by many other absorptions, some of which, without playing a part in the inactivation, may produce some other alterations in the particle (Luria, 1944). The total number of absorptions needed for one inactivating hit can be experimentally determined (Lea & Salaman, 1946; Latarjet, 1946).

All curves being similar in shape, their slope expresses the radiosensitivity of the particle: the steeper the slope, the greater the sensitivity. It has been considered that sensitivity depends on the size of the particle, and increases with it. However, recent observations on phages which have originated in lysogenic bacteria, have shown that this is not a general rule. For example (Weigle & Delbrück, 1951) the phage λ, released by *E. coli* lysogenic strain K–12, which has about the same size as the phage T_5, is much more resistant (Fig. 5).

Within a population which has been treated with UV, there is always a certain fraction which can be reactivated by exposure to visible light after adsorption to a sensitive bacterium (Dulbecco, 1949). Particles inactivated by X-rays are not photo-restorable, or, if so, only to a very small extent (Watson, 1950).

(*b*) *Virus growth in irradiated cells*

Division is one of the most radio-sensitive functions of a cell, and may be suppressed while most other functions continue. The sterilized cell maintains its metabolisms to an extent which depends on the sterilizing dose. In particular, after having received a high dose of radiation, an infected bacterium is still capable of supporting phage growth and of liberating the active particles which it contains. This holds true after X-radiation (Rouyer & Latarjet, 1946) and after UV (Anderson, 1948) as well. Virus growth within cells sterilized by radiation is also possible in the case of animal viruses, such as Shope's papilloma virus (Friedewald & Anderson, 1943). However, irradiation, which sterilizes the cell, does disturb metabolic potentialities, for virus grows slower in an irradiated cell, and yields a smaller number of new particles. For example, the burst size of phage T_2 in *E. coli* B decreases to 50% when growth takes place in bacteria which have received 9 lethal UV hits (Anderson, 1948). At the same time the latent period is about doubled. In order to suppress completely the ability of the cell to support virus growth, enormous doses of radiation must be delivered: about 100 lethal UV hits are required to reduce to half the number of B bacteria capable of liberating T_2 phage after infection (Benzer, 1952).

(*c*) *Inactivation of intracellular virus*

The following experimental findings demonstrate, in the cases which have been studied so far, that radiation suppresses the infective power of an infected bacterium (let us call the latter a complex) by directly inactivating the virus that it contains (Luria & Latarjet, 1947):

1. Immediately after single infection, before any multiplication has taken place, the survival curve of the complex is the same as that of the extracellular virus. The identity is perfect with X-rays; with UV there is a slight shift towards resistance due to shielding by the host protoplasm.
2. The survival curve of the complex is unrelated to that of the host: the same curve is obtained in the cases of T_2 mono-complexes either in bacterium B or in its radio-resistant mutant B/r.
3. Immediately after multiple infection, the survival curve of the multi-complex, in the case of X-rays, is a 'multiple target' curve with an initial plateau. Of course, some cases might occur where inactivation of the complex caused an effect on the host. The method could not then be used.

THE L.L. METHOD

(*a*) *Principle*

For a long time, viruses have been considered as degenerated bacteria with an enzymatic equipment reduced to such a degree that their multiplication has fallen under the dependence of the host's metabolisms. It was believed that, once in its host, the virus might multiply through a bi-partition process, each new particle being capable of inducing the same growth mechanism as does the initial one. Within a given cell, between infection and formation of a virus colony, several virus generations would succeed one another, and the total number of particles would increase as an exponential function of time. However, no enzymatic equipment has ever been found in a virus; it might be possible that virus multiplication is related to that of hereditary material rather than to that of monocellular organisms. In such a case there should be no reason for the preceding exponential relation to time.

It is therefore of interest to investigate how the number of particles varies in time within a given cell during the latent period. In order to number the particles, two types of experiments have been performed on bacteriophages. One consists in opening the infected cell at a given time, either by lysis (by means of another phage in excess, working through lysis from without), or by breakage by means of ultrasonics, and in counting the infective particles thus liberated. This method has disclosed very interesting results (Doermann, 1951). The other is a radiobiological method based on the preceding considerations.

1. Radiations inactivate each intracellular virus particle by a one-hit process;
2. The bacterium remains infective as long as it contains an infective particle;
3. The survival curve of multiple infected bacteria is a multiple-hit curve whose multiplicity corresponds to the number of active particles actually adsorbed by the cells.

The following experiment becomes possible:

At time zero, one infects a culture of growing bacteria (single infection); at any one time of the latent period, for instance from minute to minute, one irradiates the infected cells with several doses, and determines the survival curve at this time; this curve will resemble one of the theoretical curves (Fig. 1) corresponding to the general formula

$$y = 1-\left(1-e^{-\alpha D}\right)^{n}$$

where

y is the survival ratio
n is the multiplicity
D is the dose
α a coefficient which characterizes the radiosensitivity of the particle.

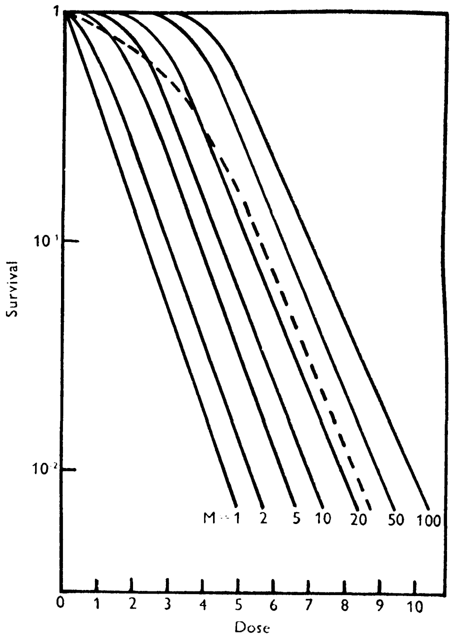

Fig. 1. Theoretical survival curves for different values of the multiplicity from 1 to 100. Dose in arbitrary units.

These curves display an initial plateau whose length is related to the number of particles (multiplicity), and a straight part which is parallel to the one-hit curve of the individual particle. The common slope of all straight parts, depending on α, reflects the radiosensitivity of the individual particle.

Any experimental curve will, by its shape, give the average number of particles per cell at the time of irradiation. Moreover, if the multiplication process is accompanied by some change in the structure of the particles which affects their sensitivity to radiation, this change will appear in the slope of the curve.

As has been pointed out already, the main advantage of this method lies in the absence of any preliminary, and possibly disturbing, treatment of the cells. Its main disadvantage is that it can be applied only to a large population, and, therefore, that it expresses only the average picture of individual events. In fact, in a bacterial culture, even as homogeneous as possible, phage multiplication is likely to proceed at varying rates, leading to a wide distribution of the phage yields from individual cells (Delbrück, 1945). Not all bursts occur at the same time; some cells yield few particles whereas others yield several hundreds. This wide distribution might somewhat distort the curves from their theoretical shape. For instance, on Fig. 1 is plotted a composite curve, established by Benzer (1952), assuming a population consisting of equal numbers of cells having the various multiplicities as shown in the figure. By extrapolating to zero dose the asymptotic slope of such a curve, it is possible, in principle, to determine the average multiplicity.

This method, initiated by Luria and Latarjet in 1947, has been improved and used by Latarjet (1948) and by Benzer (1952). Recently, it has been applied to lysogenic bacteria by Weigle & Delbrück (1951) and by Jacob (1952).

(*b*) *Experimental procedure*

Practically, a L.L. experiment is carried out as follows (see Benzer, 1952):

1. Virus adsorption. Growing bacteria are washed, resuspended at high concentration (10^9) and kept in buffer at 37° for one hour, in order to be brought to a starved condition. Phage is added in such an amount as to give single infection. With X-rays, a few multi-complexes will not disturb the results, and a proportion of one phage particle to 10 bacteria is convenient. With UV, and in the case of large phages exhibiting multiplicity reactivation, this proportion must be smaller. After adsorption, the unadsorbed phage is eliminated by a specific antiserum or by centrifugation.

2. Growth. At time zero, nutrient medium at 37° is added to the suspension of infected cells. Growth starts in all cells immediately.

3. Irradiation. At a given time, growth is suddenly stopped in all cells by chilling, and irradiation is practiced on several samples for several doses.

4. Platings. Aliquots of all irradiated samples are seeded for plaque counts, to determine the fraction of complexes whose infectivity has survived the irradiation.

(*c*) *Results*

1. *Phage T_7*—T_7 is a spherical particle, 45 mμ in diameter; its organization seems relatively simple: no tail, probably no membrane; it does not exhibit multiplicity reactivation after UV; one still does not know if it gives genetic recombinations. At 37°, its latent period in *E. coli* strain B grown in broth is 12 minutes, and the average burst size is 150 particles per infected bacterium.

Benzer (1952) has determined a set of survival curves for T_7-B monocomplexes UV-rayed at intervals during the latent period (Fig. 2). It will be observed that the curve remains unchanged—and the same as for free phage—for three minutes. As time goes on, the curves become multiple-target in character, the average multiplicity continually increasing while the final slope changes only slightly. The exact shapes of these curves may be explained by the distribution of the number of targets, which may be expected as a consequence of different rates of phage multiplication in different cells (see above). This distribution and the subsequent distortion of the curves from their theoretical aspect makes it impossible to determine the kinetics of multiplication. The curves enable us only to discern (*a*) an increase with time of the average number of targets per cell, each target having the same UV sensitivity as a T_7

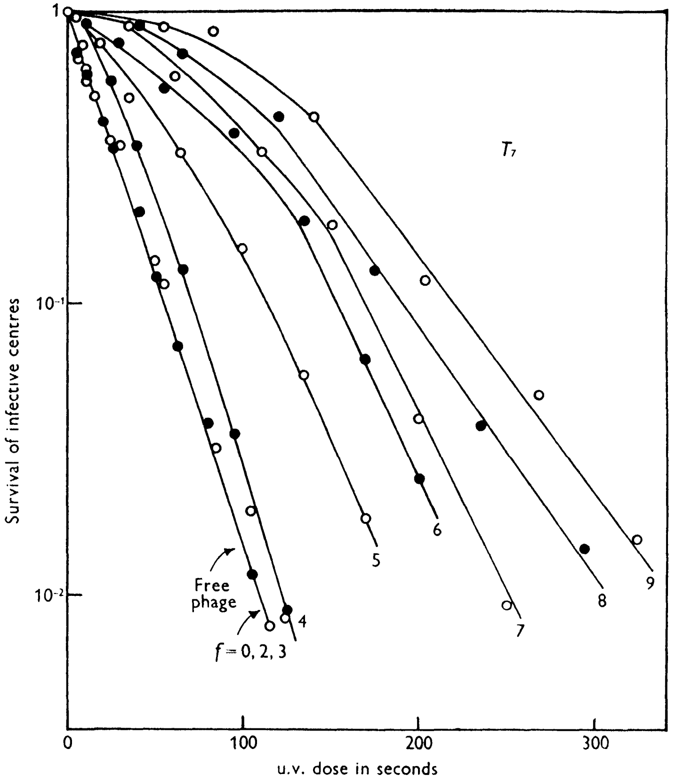

Fig. 2. UV-survival curves of T_7 mono-complexes (Benzer, 1952).

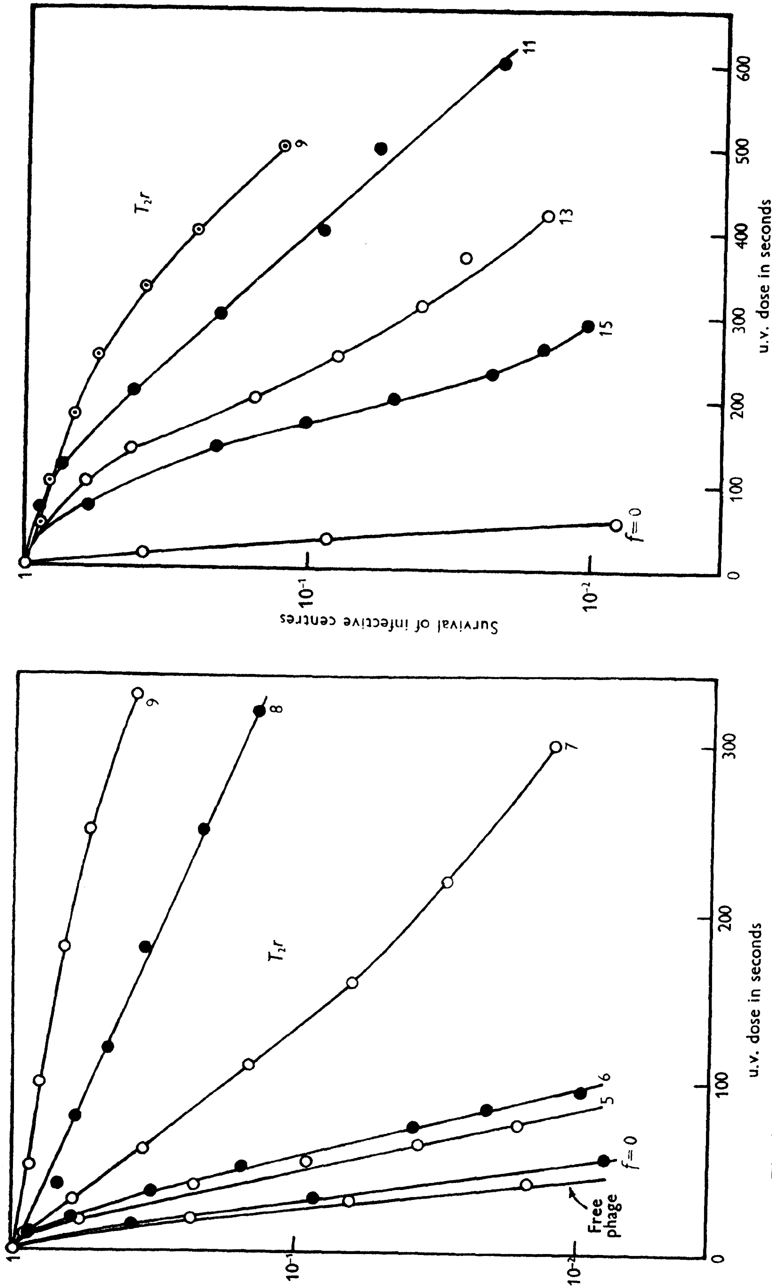

Fig. 3. UV-survival curves of T_2 mono-complexes; (*a*) from zero to 9 minutes; (*b*) from 9 minutes to 15 minutes. (Benzer, 1952.)

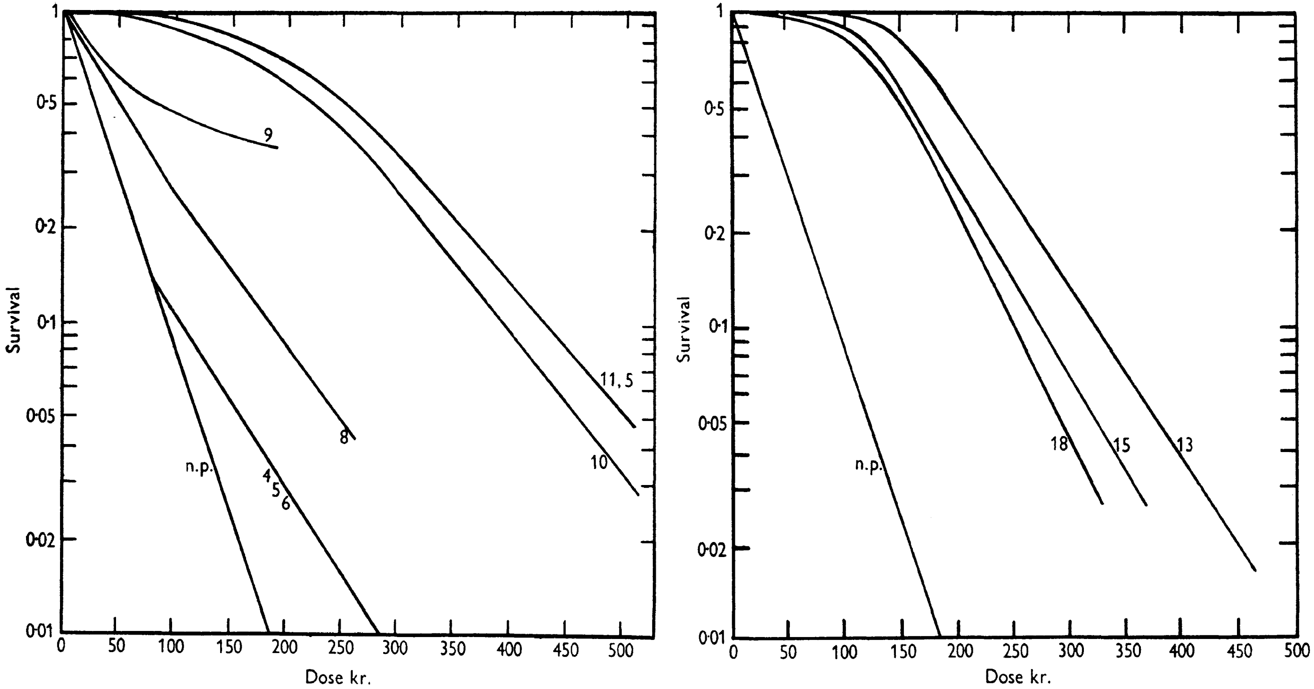

Fig. 4. X-survival curves of T_2 mono-complexes; (*a*) from zero to 11·5 minutes; (*b*) from 13 minutes to 18 minutes. Curve n.p. is that of extracellular T_2. (Latarjet, 1948.)

particle; (*b*) the existence of multiple intracellular entities at a time when fully infective phages are not yet detectable with the Doermann method.

2. *Phage T_2*—T_2 is a large and complex particle with a head 80 × 60 × 60 mμ, surrounded by a membrane, and a tail 120 × 20 mμ. It exhibits multiplicity reactivation after UV, and gives genetic recombinations with its mutants, with T_4 and with T_6. At 37°, its latent period in B is 21 minutes, and its average burst size is about 100.

The L.L. curves obtained with T_2, either with UV-rays (Luria & Latarjet, 1947; Benzer, 1952) or with X-rays (Latarjet, 1948), are shown on Fig. 3 and 4. They show that the multiplication of this phage follows a very complex process whose development can be tentatively divided into three periods of about the same duration, separated by two critical points.

First period: first 7 minutes. At time zero, the curve is the same as that of the naked phage, except that, with UV, it shows a slight increase (by 20%) of resistance. This increase may be due to shielding of UV by the bacterial protoplasm. During this first period, the evolution of the curves with UV-rays is entirely different from that observed with X-rays With X-rays, the resistance of the complexes to radiation remains constant, at least for low doses. For doses higher than 80 kiloroentgens, i.e. for survival below 10%, the remaining complexes at 5 and 6 minutes appear to be a little more resistant than the extracellular virus, as if the process which, during the second period, increases the resistance in most of the complexes, had started earlier in a few per cent of them.

With UV-rays the resistance increases continuously, the curves remaining essentially exponential. As pointed out by Benzer: 'The smallness of the bend (at large doses) in the curve taken at 7 minutes is very significant, since it implies that, at least up to this point (one-third of the way into the latent period), development has progressed rather uniformly in all infected cells.'

In agreement with the X-ray results, less than 10% of the cells, at this moment, are one minute in advance upon the others.

Second period: from 7th to 12th minute. From 7 minutes on X- and UV-curves run parallel. Resistance increases very fast. For two minutes, the curves remain of the one-hit type, with no plateau. Almost suddenly, between 9 and 10 minutes, an entirely new picture appears with a greatly increased resistance, a downward concavity and initial plateau which feature multiplicity. At 11 minutes, the resistance of the complexes to high doses has reached a maximum; the slope of the straight part of the curve has markedly decreased if compared with that of the naked particle.

Third period: from 12 minutes to burst. From this time on, the survival curve retains a definite multiple-hit type, which, especially in the X-ray experiments, displays a long plateau, a downward concavity and a straight part. As time goes on, the curve keeps its shape but the sensitivity to radiation increases slowly until the end of the latent period. At 18 minutes its straight part is parallel to the curve of the naked phage.

DISCUSSION OF THE L.L. CURVES

We shall now discuss these results according to their radiobiological significance and in relation to what has been found by others on the same virus or on some other viruses by different techniques.

(*a*) During the first 7 minutes, the X-ray curve of the complexes remains almost identical with that of the naked virus. This means that: (i) the infective virus particle remains within the cell in such a state that its X-ray sensitivity is unchanged; it remains radiologically intact, whether broken into sub-units or not; (ii) this virus remains unique; no multiplication takes place.

From Doermann's experiments, we know that during this early stage of the latent period, the particle cannot be recovered in an infective state by disruption of the cell. Delbrück & Luria (1942) have shown that in mixed infection with T_1 and T_2 in such conditions that T_2 completely suppresses the multiplication of T_1, the infecting particle of T_1 is not found in the final burst of the bacterium. These facts have contributed to show that, like other viruses such as influenza, vaccinia, ectromelia, psittacosis, etc., the bacteriophage, as soon as it enters the cell, takes on a non-infective aspect, a so-called 'masked form'. The L.L. experiment shows that such *a transformation into a masked non-infective form does not alter the X-ray sensitivity, i.e. the radiosensitive material of the particle.*

(*b*) The slight and continuous increase in resistance to UV which takes place during the first few minutes are due to changes either in the particle or in the surrounding cell protoplasm. Theoretically, it is not impossible that some change in the particle may increase the resistance to UV while leaving that to X-ray the same. However, we have evidence for bacterial syntheses of UV-absorbing material immediately after infection (Luria & Human, 1950). From Cohen's experiments (1949) the synthesis of proteins starts immediately, whereas that of DNA starts only at about the 7th minute. Thus, the early increase in UV-resistance of the complex seems to be due to shielding of the particle by newly-formed nucleoprotein material, such as purine and pyrimidine bases (without nucleotides), this material being probably devoted to the building of future particles. Such synthesis does not modify the resistance to X-rays.

It appears therefore that the first events which follow infection are:

(i) the transformation of the virus into a masked form, which implies a minor change and does not alter the material continuity of the particle; (ii) contrasting with this minor change, important metabolic deviations in the host, with synthesis of proteins. Such deviations imply the immediate mobilization of powerful enzymatic systems which would be responsible for other early effects such as: destruction of the chromatic bodies of the cell (Luria and Human, 1950), strong capacities to exclude other virus particles adsorbed by the cell (Henle & Henle, 1947; Lesley *et al.*, 1951; Dulbecco, 1952).

(*c*) Around 7 minutes a process starts which increases rapidly both X- and UV-resistance of the complex, without changing the one-target shape of the curve. Within 2 minutes, a 12 times increase in resistance is achieved, while the infective particle still remains unique. The measurements on optical density and nucleic acid content of the cells during this period (Luria & Human, 1950; Cohen & Arbogast, 1950) reveal increases which are far too small to account by screening for this resistance change. Benzer (1952) has suggested the following interpretation:

'A T_2 particle, after adsorption to a sensitive bacterium, must undergo a series of successive steps A→B→C, etc., in the course of reproduction. Each of these steps has a certain cross section for being blocked by UV. Blockade of any one of these steps prevents normal development and causes inactivation of the phage. At time zero, the total cross section of the phage is therefore the sum of these individual cross sections, and the survival curve is exponential. As development proceeds, the steps which have already been passed are no longer needed, and the effective cross section decreases progressively, while inactivation of the remaining steps retains the characteristics of a one-hit phenomenon.'

As to the nature of the critical event which occurs at minute 7, it is very tempting to correlate it to the beginning of the synthesis of DNA which starts at the same moment (Cohen, 1949).

(*d*) Around 9 minutes, in both UV and X, a new phenomenon transforms the survival curve into a multiple-hit one, with a definite plateau and a straight part whose slope is weaker than that of the naked virus curve. This indicates that (i) some units are multiplying very fast and are already numerous at 10 minutes; (ii) these units are more resistant to radiation (probably smaller) than the extracellular particle. The multiplicity increases rapidly and reaches its maximum around 12 minutes with about 100 potentially virulent units whose X-ray sensitivity is about half that of the initial particle. This picture suggests several considerations, and reveals two important facts:

(i) If, as pointed out by Luria (1950) 'all growth and reproduction should ultimately be traceable to replication of specific chemical configurations by an essentially discontinuous appearance of discrete replicas', *in the course of the 21 minutes long intracellular life of* T_2*, such ultimate replication takes place during about 4 minutes only* (from 9 to 12 minutes). Such rapidity is not surprising and appears as a general feature. It has been shown in cells of a high evolutionary development that the main syntheses of genetic material take place during the interphase—not during the prophase—at such a rate that they can be achieved within a few minutes, while the whole mitotic cycle may take several hours (Lison & Pasteels, 1951; Howard & Pelc, 1951).

(ii) The X-ray experiments definitely prove that *the virus does not duplicate within the cell under its extracellular form.* In other words, what replicates is different from the infecting particle, the difference resulting mainly from the changes occurring in the two minutes (7–9) which immediately precede replication. This fact has been recently confirmed by Doermann's experiments on breakage of cells mixedly infected with T_2H_r and T_2H_h (1951), which, according to Hershey & Rotman (1949), give 2 to 3 per 100 recombinants:

'If one artificially breaks the cell early . . . when there is a small number of phage particles per cell, the percentage of recombinants is about as high as is found when cells are allowed to lyse normally. On the hypothesis of binary fission, these early recombinants would necessarily grow into clones. Hershey & Rotman's evidence is unequivocal that no clones are found. One must therefore conclude that the phages of the T even series do not multiply by binary fission, and further that the first completed phage particles are not themselves the parents of those viruses which appear later within the same cell.'

(*e*) What is the nature of the units which multiply so rapidly from about the 9th to the 12th minutes, and which, according to the curves, seem smaller than the initial particle? Are we dealing with a *unique* fraction, some kind of genetic nucleus, of that particle, which, around 12 minutes, would give 100 identical nuclei; or with *multiple* sub-units more or less capable of independant multiplication? The L.L. curves would be the same in both cases, and cannot, therefore, decide between them.

A second radiobiological method can bring here some new information. It consists in studying the photo-restoration of complexes which have been UV-rayed at various times of the latent period, or, in other words, in comparing to the preceding L.L. curves obtained in the dark, the L.L. curves obtained maximum photo-restoration. (Let us notice that this study cannot be carried out with X-rays, since X-inactivation of the phage is not photo-restorable). Used by Dulbecco and Benzer, this method has already yielded interesting results (personal communication) which will not be quoted here since they are still unpublished. Let us only say that they bring some evidence for the multiplication of a single nucleus rather than of multiple sub-units. A small phage such as T_7 would be entirely constituted with this nucleus, hence the simple aspect of the L.L. curves, without any change in the slope (Fig. 2). A large phage such as T_2 would contain such a nucleus A and a second fraction B, both A and B being radiosensitive. A would multiply first from 1 to 100 within about 4 minutes (9–12) which would correspond to a replication time of about 35 seconds. A would therefore predominate during this period in the radiobiological picture, with its smaller cross-section and higher resistance to radiation. Synthesis of the B fractions would occur later during the third period, leading to the progressive increase in the average cross-section which appears in the L.L. curves from the 12th minute on.

(*f*) The L.L. curves show a second critical point between 12 and 13 minutes which corresponds to a reversion in the radioresistance of the complexes and which marks the end of the multiplication. At this moment, the average cell contains 100 'units' which are twice as resistant as the naked phage particle. From this time on, this average multiplicity of 100 does not change, but the individual resistance of the units progressively decreases, and, at the end of the latent period reaches, as expected, that of the extracellular virulent particle.

The existence of a second critical point around 12 minutes which marks the end of the multiplication has been confirmed by Foster's experiments on the same phage T_2 (1948): if one adds proflavin or 5-methyl-tryptophane to the growing medium, one inhibits multiplication of the phage—an inhibition which is counteracted by an excess of DNA. But this phenomenon occurs only if the chemical is added during the first 12 minutes of the latent period. This proves that at this moment the synthetic steps which are sensitive to this inhibition are completed.

Electron microscope observations of growing B-T_2 complexes (Wyckoff, 1948) confirm the radiobiological picture: it is only by the middle of the latent period that some particles can be seen within the bacterial protoplasm. Small at first, they rapidly increase in size and in number, and invade the whole cell. The typical tadpole aspect of the adult virus appears only at the end of the latent period.

By breaking growing B-T_2 complexes, Doermann (1951) succeeded in counting the infective intracellular T_2 particles at several times, and contributed very valuable information on the kinetics of maturation of the virus. The first infective particles appear by the middle of the latent period (11 minutes); further, their number increases with time. This confirms the fact that the units which multiply between 9 and 12 minutes are not infective and that, in order to become so, they must undergo some maturation which takes place after multiplication. This maturation ends the masked state of the virus. This unmasking process occurs only during the second half of the latent period.

The idea that the virus multiplies as a non-infective immature form and undergoes later maturation which endows it with infective power, is consistent with several cytological observations on animal viruses such as Psittacosis, Lymphogranuloma, Vaccinia, Ectromelia (see discussion in Bauer, 1949; Horsfall, 1949). In this respect, studies carried out on the influenza virus (Henle *et al.*, 1947; Hoyle, 1948) are particularly significant. During growth, at least two varieties of particles appear: one is complete, immature, non-infective, small in size, and capable of agglutinating blood-red cells; it probably corresponds to the small antigenic constituents which appear in the infected tissues long before the second variety, that is the classical mature virus of larger size. Just as T_2, the influenza multiplies as a form which is almost entirely non-infective: only 10^{-4} particles would be infective at that time. Thus, in the case of influenza, multiplication is initiated by the virulent mature particle only, but the particles which multiply are smaller, immature and non-infective. It seems that we have here the same situation as in the case of T_2. Similarly, it seems that the Shope papilloma virus multiplies as an immature, antigenic and non-infective particle. In the cotton-tail wild rabbit this multiplication would be followed by maturation into infective complete particles; this maturation would not take place in the domestic rabbit (see Shope, 1937).

As regards the maturation of T_2 particles, it should be pointed out that from Cohen's (1949) and Doermann's (1951) independent investigations, there appears a striking parallel in the accumulation of newly synthesized DNA in the cell and the subsequent increase in mature phage particles. Therefore the maturation process which endows the particle with infective power seems to depend on DNA. The same dependence has been observed by Markham *et al.* (1948) on the crystalline mosaic turnip yellow virus: by centrifugation one can separate two constituents, (i) the classical virus, an infective nucleoprotein, and (ii) a non-infective particle, half as small and devoid of nucleic acid. Both particles react to the same anti-virus serum; both have the same electrophoretic mobility; both crystallize in the same system and their proteins contain the same amino acids in the same proportions. The mature infective particles differ from the others by the presence of DNA.

THE CASE OF LYSOGENIC BACTERIA

Among their hereditary material, lysogenic bacteria contain a provirus which usually does not pullulate. Under certain influences this provirus originates an important development leading to the formation of several hundreds of virus particles per cell which are liberated into the medium by lysis of the cell (Lwoff & Gutmann, 1950). In particular when the bacteria are grown in certain conditions, a suitable UV-irradiation 'induces' this virus synthesis in almost all cells (Lwoff *et al.*, 1950). X-irradiation achieves the same induction with an even higher efficiency (Latarjet, 1951).

It is of interest to know how many provirus particles the bacterial cell normally contains. Here again, the L.L. method is capable of giving an approximate answer to this question when suppression of phage growth is due to an effect on the prophage. A L.L. experiment can be carried out on lysogenic bacteria thanks to the fact that induction is more radiosensitive than inactivation, i.e. the UV doses which induce the synthesis of the virus are small if compared to those which inactivate that virus.

Let us consider, for example, *E. coli* lysogenic strain K-12 which, through spontaneous or induced lysis, liberates a phage λ active on *E. coli* strain S (Weigle & Delbrück, 1951). As has been already pointed out, this carried phage λ is much more radioresistant than other phages of the same size active on K-12, such as T_5 (Fig. 5, curves 4 and 1). A known number of K-12 bacteria are irradiated with increasing doses of UV. After each dose, an aliquot is seeded on nutrient agar with an excess of the indicator bacteria S. Under certain conditions, each K-12 cell in which the synthesis of the virus λ takes place gives after lysis a clear plaque on the plate. Without irradiation, spontaneous lysis occurs in 5×10^{-5} cells per generation. When the UV dose increases, the induction phenomenon is produced in more and more cells, approaching 100% (Fig. 5, dotted curve 5). After a plateau, the curve falls down as a straight line which is parallel with the inactivation curve (4) of free phage λ. Such parallelism which has been found in the other lysogenic bacteria so far studied, means: (i) that suppression of the induction by high UV doses is due to inactivation of the prophage; (ii) that the prophage particle has the same radiosensitivity as the particle of the extracellular adult phage. The plain curve 5 appears therefore as a L.L. curve which brings an estimate of the average number of prophage particles per cell

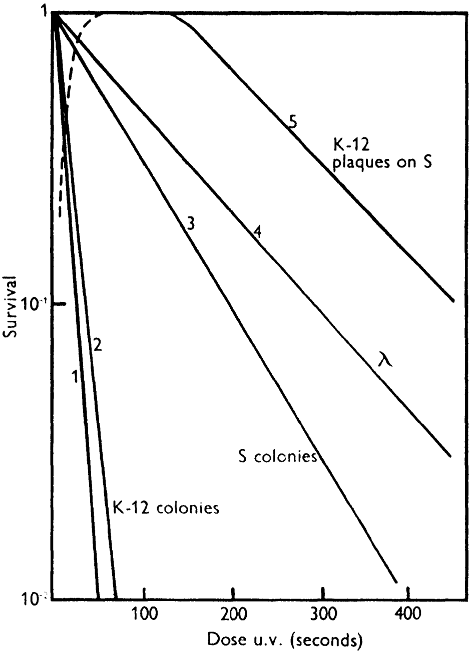

Fig. 5. UV-survival curves of free phages T_5 (curve 1) and λ (curve 4), of bacteria K-12 (curve 2) and S (curve 3); survival of K-12 as regards its ability to liberate active λ (curve 5). (Weigle and Delbrück, 1951.)

at the moment of irradiation. In the present case, this number is equal to about 3.

The same experiment, carried out by Jacob (1952) on lysogenic *P. pyocyanea* strain P 13/8A, gave the same result, that is an average number of 3 prophage particles per cell.

REFERENCES

Anderson, T. F. (1948). The growth of T_2 virus on ultraviolet-killed host cells. *J. Bact.*, **56,** 403.

Bauer, D. J. (1949). Multiplication of the viruses. *Nature*, **164,** 767.

Benzer, S. (1952). Resistance to ultraviolet light as an index to the reproduction of bacteriophage. *J. Bact.* (in press).

Cohen, S. S. (1949). Growth requirements of bacterial viruses. *Bact. Rev.*, **13,** 1.

Cohen, S. S. & Arbogast, R. (1950). The mutual reactivation of T2r virus inactivated by UV light and the synthesis of desoxyribosenucleic acid. *J. Exp. Med.*, **91,** 637.

Delbrück, M. (1945). The burst size distribution in the growth of bacterial viruses. *J. Bact.*, **50,** 131.

Delbrück, M. & Luria, S. E. (1942). Interference between bacterial viruses. *Arch. Biochem.*, **1,** 111.

DOERMANN, A. H. (1951). Intracellular phage growth as studied by premature lysis. *Fed. Proc.*, **10**, 591.

DULBECCO, R. (1949). Reactivation of ultraviolet inactivated bacteriophage by visible light. *Nature*, **163**, 949.

DULBECCO, R. (1952). Mutual exclusion between related phage. *J. Bact.* (in press).

FOSTER, R. A. (1948). An analysis of the action of proflavine on bacteriophage growth. *J. Bact.*, **56**, 795.

FRIEDEWALD, W. F. & ANDERSON, R. S. (1943). The effects of Roentgen rays on cell-virus associations. *J. Exp. Med.*, **78**, 285.

HENLE, W. & HENLE, G. (1947). The effect of UV irradiation on various properties of influenza viruses. *J. Exp. Med.*, **85**, 347.

HENLE, W., HENLE, G. & ROSENBERG, E. B. (1947). The demonstration of one-step growth curves of influenza viruses through the blocking effect of irradiated virus on further infection. *J. Exp. Med.*, **86**, 423.

HERSHEY, A. D. & ROTMAN, R. (1949). Genetic recombination between host range and plaque-type mutants of bacteriophage in single bacterial cells. *Genetics*, **34**, 44.

HORSFALL, F. L. (1949). Viral multiplication. *Fed. Proc.*, **8**, 518.

HOWARD, A. & PELC, S. R. (1951). Synthesis of nucleoprotein in bean root cells. *Nature*, **167**, 599.

HOYLE, L. (1948). The growth cycle of influenza virus A. *Brit. J. Exp. Path.*, **29**, 390.

JACOB, F. (1952). Effects de la carence en aliment carboné sur l'induction d'un Pseudomonas pyocyanea lysogène. *Ann. Inst. Pasteur* (in the press).

LATARJET, R. (1946). L'effet biologique primaire des radiations. *Rev. Canad. Biol.*, **5**, 9.

LATARJET, R. (1948). Intracellular growth of bacteriophage studied by Roentgen irradiation. *J. Gen. Physiol.*, **31**, 529.

LATARJET, R. (1951). Induction, par les rayons X, de la production d'un bactériophage chez *B. megatherium* lysogène. *Ann. Inst. Pasteur* **81**, 389.

LATARJET, R. & MORENNE, P. (1951). Inactivation d'un bactériophage par un rayonnement ultraviolet de très faible intensité. *Ann. Inst. Pasteur*, **80**, 220.

LATARJET, R. & WAHL, R. (1945). Précisions sur l'inactivation des bactériophages par les rayons ultraviolets. *Ann. Inst. Pasteur*, **71**, 336.

LEA, D. E. & SALAMAN, M. H. (1946). Experiments on the inactivation of bacteriophage by radiations, and their bearing on the nature of bacteriophage. *Proc. Roy. Soc. B*, **133**, 434.

LESLEY, S. M., FRENCH, R. C., GRAHAM, A. F. & VAN ROOYEN, C. E. (1951). The breakdown of T2r+ bacteriophage upon infection of its host *Escherichia coli*. *Canad. J. Med. Sci.*, **29**, 128.

LISON, L. & PASTEELS, J. (1951). Etudes histophotométriques sur la teneur en acide desoxyribose nucléique des noyaux au cours du développement embryonnaire chez l'oursin. *Arch. Biologie*, **62**, 1.

LURIA, S. E. (1944). A growth-delaying effect of UV radiation on bacterial viruses, *Proc. Nat. Acad. Sci.*, **30**, 393.

LURIA, S. E. (1947). Reactivation of irradiated bacteriophage by transfer, of self-reproducing units. *Proc. Nat. Acad. Sci.*, **33**, 253.

LURIA, S. E. (1950). Bacteriophage: an essay on virus reproduction. *Science*, **111**, 507.

LURIA, S. E. & HUMAN, M. L.(1950). Chromatin staining of bacteria during bacteriophage infection. *J. Bact.*, **59**, 551.

LURIA, S. E. & LATARJET, R. (1947). Ultraviolet irradiation of bacteriophage during intracellular growth. *J. Bact.*, **53**, 149.

LWOFF, A. & GUTMANN, A. (1950). Recherches sur un *Bacillus megatherium* lysogène. *Ann. Inst. Pasteur*, **78**, 711.

LWOFF, A., SIMINOVITCH, L. & KJELDGAARD, N. (1950). Induction de la production de bactériophages chez une bactérie lysogène. *Ann. Inst. Pasteur*, **79**, 815.

MARKHAM, R., MATTHEWS, R. E. & SMITH, K. M. (1948). Specific crystalline protein and nucleoprotein from a plant virus having insect vectors. *Nature*, **162**, 88.

ROUYER, M. & LATARJET, R. (1946). Augmentation du nombre de bactériophages en présence de bactéries stérilisées par irradiation. *Ann. Inst. Pasteur*, **72**, 89.

SHOPE, R. E. (1937). Immunization of rabbits to infectious papillomatosis. *J. Exp. Med.*, **65**, 219.

WATSON, J. W. (1950). The properties of X-ray-inactivated bacteriophage. *J. Bact.*, **60**, 697.

WEIGLE, J. J. & DELBRÜCK, M. (1951). Mutual exclusion between an infecting phage and a carried phage. *J. Bact.*, **62**, 301.

WOLLMAN, E. & LACASSAGNE, A. (1940). Evaluation des dimensions des bacteriophages au moyen des rayons X. *Ann. Inst. Pasteur*, **64**, 5.

WYCKOFF, R. W. G. (1948). The electron microscopy of developing bacteriophage. *Biochim. Biophys. Acta*, **2**, 27.

DIVALENT METALS IN PHAGE PRODUCTION

PAUL FILDES[1], D. KAY[2] AND W. K. JOKLIK[3]

Sir William Dunn School of Pathology, Oxford University

A number of authors have implicated calcium (or other divalent metals) in the successful action of various phages.

For instance Stassano & de Beaufort (1925) found that citrate inhibited the action of certain Shiga phages implicating Ca indirectly. Bordet & Renaux (1928), however, showed that a decalcified medium inhibited the growth of a Shiga phage until Ca was added, while a normal broth could be inactivated with oxalate.

Andrewes & Elford (1932) also noted that citrate prevented the multiplication of coli phage, but adsorption was not affected and the infected bacteria were unable to multiply.

Burnet (1933) used citrate inhibition in the classification of coli-dysentery phages.

Gratia (1940) found that Ca was indispensable for the adsorption of certain phages and a similar view was taken by Delbrück (1948) of a mutant of the T4 coli phage although in his case adsorption was only increased some 5 times. On the other hand Wahl (1946) found that coli phage adsorbed normally without Ca, but multiplication only took place in a narrow zone of concentrations.

In 1949 Adams showed that the T5 coli phage depended on added Ca for effective lysis, but that adsorption and the lethal action on the infected bacteria did not.

Kay and Fildes (1950) worked with a typhoid phage and found that gross lysis and multiplication did not occur in the absence of added Ca, but nevertheless a minor degree of multiplication did take place. They noted that a so-called Ca-free medium must necessarily contain Ca derived from the phage lysate used in the experiment.

Beumer & Beumer-Jochmans (1951) restudied the dysentery phage used by Bordet (1926). They found that this phage failed to multiply in a Ca-free medium because no adsorption took place. Neither did inactivation occur on mixing a bacterial extract with the phage. Both

[1] Director, Virus research unit, British Empire Cancer Campaign.

[2] Grantee of the Medical Research Council.

[3] Australian National University Scholar.

actions however took place in the presence of Ca. Another phage acting in the same bacterium was indifferent to Ca.

Shew (1949) worked with a phage operating on a lactic streptococcus and found that while lysis in broth was poor, in milk it was active. This result was ascribed to the higher content of Ca in milk. The effect was not on adsorption.

From this short summary it will be seen that some dysentery, coli, typhoid bacilli and lactic streptococci phages depend to some extent on Ca for their effective multiplication, but that the point of action in the cycle is said to be variable.

It is difficult to determine whether in these cases Ca is an absolute requirement or whether it is merely a stimulant of a process which proceeds more slowly without the addition. This is due to the fact that few authors have described any special precautions for eliminating Ca impurities from their media and apparatus.

TYPHOID PHAGE

Much of our work has been carried out with a typhoid phage in which Ca is at least highly important (Kay & Fildes, 1950). We have since made great efforts to avoid contamination with Ca in an attempt to show that no multiplication of phage takes place in the absence of this metal, but our effort to prove a negative has been disturbed by the fact

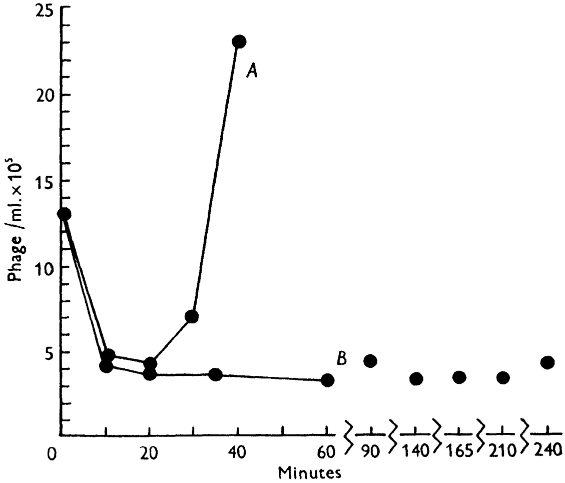

Fig. 1. *The Effect of Ca on bursts with Typhoid Phage.*
At time 0. 10^8 bacteria/$1 \cdot 3 \times 10^6$ phage/ml. A contained M/4000 Ca
At time 10. Both diluted 1/1000, A *plus* M/4000 Ca

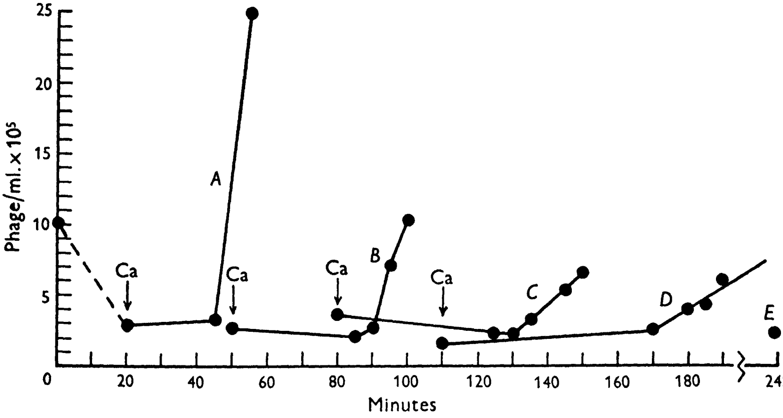

Fig. 2. *The Effect of delayed addition of Ca on bursts with Typhoid Phage.*
At time 0. 10^8 bacteria/1·0 × 10^6 phage/ml. No Ca
At time 10. Dil. 1/1000 without Ca
At time 20 and onwards, samples removed to Ca to make M/4000.
At time 245. Point E indicates the remaining material without Ca

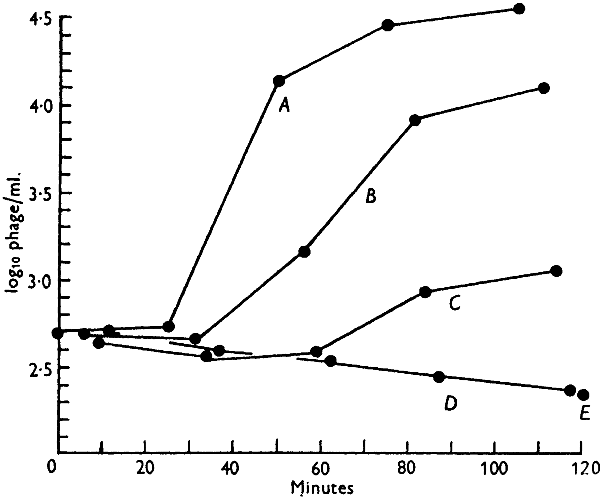

Fig. 3. *The Effect of Ca concentration on bursts with Typhoid Phage.*
Previous to time 0. 10^8 bacteria/1·5 × 10^6 phage/ml. without Ca
Adsorbed 15 min.; diluted 1/100 without Ca; centrifuged; washed and made up to volume ≡ 1/100 without Ca
At time 0. Five identical samples at 37°. M/3000 Ca to A.
At time 6. M/17500 Ca to B.
9. M/43740 Ca to C.
12. M/109340 Ca to D.
No Ca in E.

that biologically active concentrations cannot be detected and absence can never be achieved because the phage preparation itself necessarily contains Ca which cannot at present be eliminated.

All experiments referred to in this paper were made with phage and bacteria grown on a simple glucose-ammonia mixture.

Fig. 1 shows the result of an experiment in which M/4000 Ca was added at time 0 to one fraction A of a typhoid phage/bacterium mixture and none to fraction B. In the presence of Ca the burst occurred between 20 and 30 minutes; in its absence no burst was observed during four hours.

The failure to burst in this experiment is due to the absence of Ca. This can be proved by allowing adsorption to take place in the absence of Ca and adding Ca as in Fig. 2. Here it is seen that after each addition of Ca a burst occurred. The tendency for the incubation period to be prolonged and the burst size to be reduced after delayed additions is no doubt due to irreversible changes which take place in the absence of Ca.

Fig. 3 shows that the size and time of burst is related to Ca quantitatively; as the Ca concentration is reduced so is the burst size reduced and the burst delayed. In this case after adsorption had taken place for 15 minutes in the absence of Ca, the mixture was centrifuged and the washed deposit only used. Thus all the active centres were derived from adsorbed phages which were capable of producing phages on Ca-containing agar but not of bursting in the liquid medium until Ca was added.

All these experiments show that in the typhoid phage system used adsorption can take place in the absence of Ca, but that these adsorbed phages cannot give rise to bursts until Ca is added either on plating or in the liquid. Further the average yield of phage is related to the concentration of Ca added.

It is admitted that results so conclusive as those illustrated are not invariable. Minor bursts outside the limit of experimental error are not infrequent in the absence of added Ca, but these are certainly reduced in number by addition of M/1000 citrate. This procedure would reduce the concentration of calcium ions present as chance impurities.

Further evidence of the action of Ca in the typhoid system is obtained from burst size experiments on individual infected bacteria.

In these adsorption was allowed to take place (10^8 bacteria/10^6 phage) in the absence of Ca for 15 minutes. After centrifuging, the washed deposit only was used. This was highly diluted so that less than 1 phage-infected bacterium was present in each ml. of a 60 ml. batch. Sixty 1 ml. samples were then incubated in the presence of Ca and in its absence.

To these latter M/1000 citrate was added.

In the presence of M/3500 Ca incubation continued for 1 hour before the whole samples were plated.

It was found that 21 of 60 samples were infected. Fourteen of these contained between 192 and 49 phages with an average of 91 per cell assuming that all were single infections. Two contained only three phages and five only one.

In the absence of Ca incubation was continued for 3 hours to cover a possibly prolonged burst time suggested by the experiment illustrated in Fig. 3.

In this case 28 samples were infected. Six of these contained between 5 and 2 phages with an average of 3. The remaining 22 contained 1 phage only.

In these experiments it is assumed that practically every infected tube contained at first only one bacterium infected by one phage; for instance in the experiment with Ca only 3, producing over 100 phages each, might according to the Poisson distribution contain more than one.

Thus it may readily be concluded that those tubes which later contained 49 or more had undergone bursts. Further those tubes which had only one might be supposed to have failed to burst. A difficulty, however, arises with the 2 tubes in the Ca experiment which contained 3 phages and with the 6 tubes in the absence of Ca which contained 5 to 2. According to the Poisson distribution it is highly improbable that these tubes started with 5 to 2 phages. It is more likely that bursts occurred in these tubes producing only 5 to 2 phages.

It seems to be a reasonable conclusion from these experiments that the burst size, i.e. the phage yield is governed by the concentration of Ca available to the system after adsorption has taken place and that in an ideal case where no Ca was present the system would remain in the inactive state in which no phage is demonstrable.

This, however, is a generalization which only applies in a homogeneous system. It is clear that a system which produces from 2 to over 100 phages per burst in the same concentration of Ca, is not homogeneous.

The point at issue, however, is not whether the system always produces phage when Ca is present, but whether it never produces phage in its absence. It seems probable that in an ideal case this would be so.

PHAGE T5

Since Ca has no demonstrable effect on adsorption in the typhoid system, it follows that its action must take place after this event and before rupture of the bacterium, i.e. it may catalyse the solution or disappearance of the adsorbed phage, direct this in such a way that subsequent synthesis can take place or catalyse the synthesis.

Experiments with the T5/coli B system bear upon this matter. In this system Ca is also highly important but not so specifically required as in the typhoid system. In this latter Mg has no demonstrable effect, but with T5 both Sr and Mg are active but no other divalent metal (Fig. 4). Since Coli B requires Mg in a higher concentration for growth than does typhoid, it follows that with Ca experiments there is always a higher blank due to Mg. For instance in the best case observed T5 gave a burst of 410 with Ca and 6 without (Fig. 5).

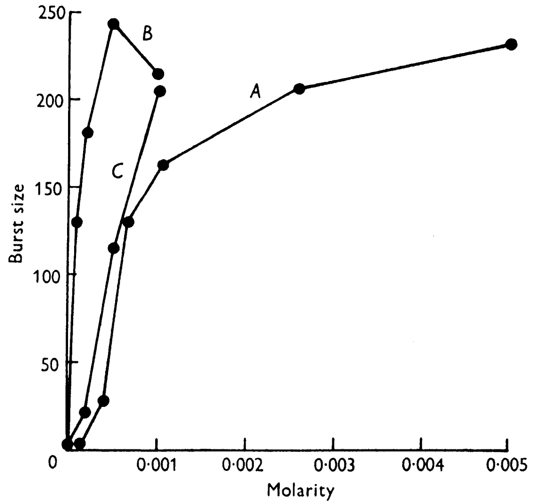

Fig. 4. *The Effect of divalent metal concentration on the burst size of phage T5.*
A = Magnesium.
B = Calcium.
C = Strontium.

T5 was used to study intracellular phage growth by artificial solution of the infected bacterium.

The process adopted made use of Doermann's (1948) method of inhibiting phage synthesis by cyanide and Maculla & Cowles' (1948) method for dissolving bacteria with strong solutions of glycine.

Cultures of bacteria were allowed to adsorb phage and then washed to remove free phage. The suspension was divided and calcium added to one portion. Samples were then plated at intervals to determine the total number of infective centres and the time and size of the burst.

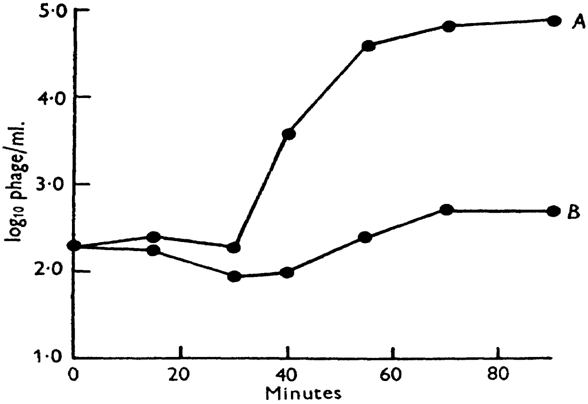

Fig. 5. *The multiplication of phage T5.*
A = Without calcium.
B = With M/2000 calcium.

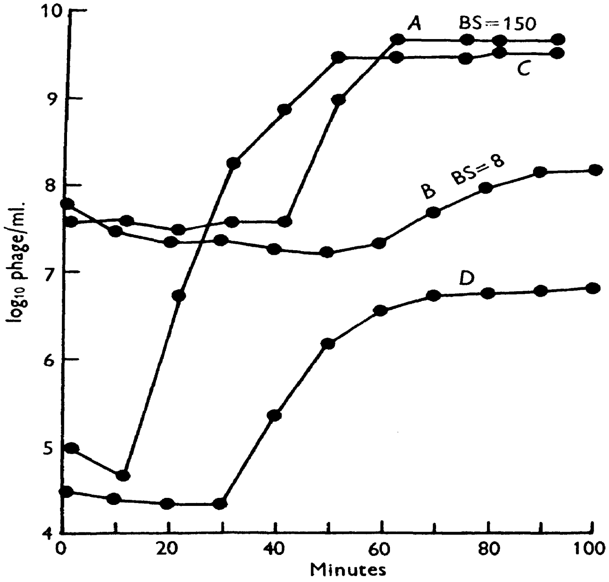

Fig. 6. *The multiplication of phage T5.*
A = Total count with M/2000 calcium.
B = Without calcium.
C = Intracellular phage with M/2000 calcium.
D = Intracellular phage without calcium.
BS = Burst size.

Simultaneously samples were added to the mixture of cyanide and glycine and plated out later for an intracellular phage count. Fig. 6 shows the effect of calcium on the total and intracellular phage both expressed as plaques per ml.

At first with or without calcium the number of infective centres was about the same but later the number with calcium far exceeded the number without owing to a much greater burst. In both cases intracellular phage was much reduced in the first few minutes. The small counts actually obtained were probably due to free phage or unlysed cells. However, with calcium present a rapid increase in intracellular phage took place beginning between 12 and 22 minutes. By 26 minutes each infective centre (curve A) contained an average of one phage particle and after 42 minutes, at which time lysis was beginning, this number had increased to 100. Without calcium, on the other hand, a much smaller increase in intracellular phage took place and at no stage did the infective centres contain an average of even one phage particle.

This experiment confirms the general view that soon after adsorption has taken place, there are no intact phages in the cell. Those adsorbed have disappeared, to reappear and increase in number later.

It also shows that Ca governs the synthesis of phage. The initial disappearance takes place without it. The experiment, however, does not distinguish between a direct or indirect action of Ca if such a distinction can be made.

PHAGE T1

In the case of T1 the requirement for divalent metals is still absolute but less particular.

Whereas a T1/coli B mixture at a multiplicity of 1/200 did not lyse in 150 minutes in the presence of 0·00008 *M* Mg, the addition of 0·00033 *M* Mg, Ca, Sr, Mn or even Ba (in a low sulphate medium) caused lysis in 90–100 minutes.

Owing to the relatively high concentrations of Mg necessary for the metabolism of the bacterium it is impossible to determine quantitatively the separate effect of this or other divalent metals on the phage production cycle.

CONCLUSION

So far as our experiments are applicable they are in agreement with those of Adams (1949).

It would appear possible to predict that, at any rate in the case of the typhoid phage used in these experiments, any mechanism which may be postulated for the synthesis of phage must include a function for the metal calcium.

REFERENCES

ADAMS, M. H. (1949). The calcium requirement of coliphage T5. *J. Immunol.*, **62**, 505.

ANDREWES, C. H. & ELFORD, W. J. (1932). The 'killing' of bacteria by bacteriophage. *Brit. J. Exp. Path.*, **13**, 13.

BEUMER, J. & BEUMER-JOCHMANS, M. P. (1951). Les exigences en calcium du phage de Lisbonne. *Ann. Inst. Pasteur*, **81**, 489.

BORDET, J. & RENAUX, E. (1928). L'autolyse microbienne transmissible ou le bacteriophage. *Ann. Inst. Pasteur*, **42**, 1283.

BURNET, F. M. (1933). The classification of dysentery-coli bacteriophages. III. A correlation of the serological classification with certain biochemical tests. *J. Path. Bact.*, **37**, 179.

DELBRÜCK, M. (1948). Biochemical mutants of bacterial viruses. *J. Bact.*, **56**, 1.

DOERMANN, A. H. (1948). Intracellular growth of bacteriophage. *Carnegie Inst. Wash. Yearbook*, **47**, 176.

GRATIA, A. (1940). Action des électrolytes sur les bactériophages et sur leur fixation par les bactéries sensibles. *Bull. Acad. Roy. Med. Belg.*, **5**, 232.

KAY, D. & FILDES, P. (1950). The calcium requirement of a typhoid bacteriophage. *Brit. J. Exp. Path.*, **31**, 338.

MACULLA, E. S. & COWLES, P. B. (1948). The use of glycine in the disruption of bacterial cells. *Science*, **107**, 376.

SHEW, D. I. (1949). Effect of calcium on the development of streptococcal bacteriophages. *Nature*, **164**, 492.

STASSANO, H. & DE BEAUFORT, A. C. (1925). Action du citrate de soude sur le principe lytique transmissible. *C. R. Soc. Biol.*, **93**, 1380.

WAHL, R. (1946). Influence de la composition du milieu sur la bactériophagie. *Ann. Inst. Pasteur*, **72**, 73.

DISCUSSION

P. FILDES: The facts relating to calcium and divalent metals in phage production by the systems referred to are given in our paper. They are an elaboration of the original discovery of Bordet and Renaux made twenty-four years ago and amply supported by a number of workers since. It is curious to me that in a symposium which has attracted so many workers in all fields, no reference has been made to calcium, although this metal appears to take some decisive part in phage production at any rate by the typhoid bacillus I have worked with.

N. W. PIRIE: We have not all neglected calcium completely in recent years: the position is rather that we have found the study difficult and hard either to repeat or to interpret. Bawden and I found (*J. Gen. Microbiol.* 1950, **4**, 482) that a tobacco necrosis virus lost its infectivity on exposure to neutral M/50 citrate but was protected from this inactivation by $CaCl_2$ or NaCl. It was also more stable in stronger citrate. At first sight this looks like a calcium effect, but it is not easy to fit all the observations into a simple picture.

P. FILDES: I also have not found it easy. I have referred in the paper to the main difficulty. There seems to be no method available for the detection of biologically active traces of calcium in these mixtures. You cannot be certain whether calcium is biologically absent or not.

D. KAY: I should like to amplify Sir Paul Fildes' remarks about the divalent metal requirement of coli phage T5. Measurements of the burst size show that calcium at 0·0005 *M* is most active in promoting phage production. The second most active metal is strontium and the least active is magnesium which at 0·005 *M* gives a burst size almost equal to that given by 0·0005 *M* calcium. No other metal has been found to be active. I would like to stress the difference between this phage and the typhoid phage which responds only to calcium on the one hand and the T1 phage which responds to Mg, Sr, Ca, Mn and Ba on the other.

The exact position at which calcium acts in the multiplication of T5 is not yet known but it is clear that adsorption is independent of calcium and that the disappearance of the infective virus from the cell contents also takes place without calcium being present. However, the appearance of new phage particles, which would begin at about 20 minutes after adsorption if calcium were present, does not take place. We may therefore tentatively assume that calcium is involved in the processes whereby the non-infective form of the virus is transformed into fully infective particles.

I have also done some experiments with phage T5 on the variation in the uracil and thymine content of the cells. Phage nucleic acid is all of the deoxyribose type and contains thymine but not uracil whereas ribose nucleic acid, which is present in the bacteria as well as DNA, contains uracil but not thymine. It was found that uracil synthesis was almost completely inhibited by phage infection. Thymine synthesis on the other hand, after a lag of about 10 minutes, proceeded at a rate somewhat greater than in uninfected bacteria.

BACTERIOPHAGE NOT A VIRUS?

A. FELIX: I think all of us will agree on at least one point: that the authors of the main contributions to the Symposium have given us an ample supply of new theories; even in the present atomic era of rapid changes, this seems to be a remarkable achievement. Some of us may also agree on another point, namely, that virologists have always been, and still are, rather a greedy species. They swallowed Rickettsia, they conquered bacteriophage, cancer is always in danger of being subjugated by them, and it is difficult to foresee where they are going to stop.

I fully agree with Dr Lwoff that it is impossible to discuss the nature of virus reproduction without attempting to give a definition of what is a virus. As a mere bacteriologist working with bacteriophage I have been and still am unable to accept the theories favoured by the leading virologists and I feel justified in refusing to consider bacteriophages as being viruses at all.

It may be of interest to quote a few sentences from the report of an earlier Symposium on this subject arranged by this Society in 1946 (at Leeds). I am reading from a reprint from *Nature* (**158**, 406), dated 21 September, 1946. 'All speakers at the discussion agreed that the degraded parasite hypothesis was almost certainly true as regards the animal viruses, at least the larger ones. As Dr F. M. Burnet of Melbourne pointed out, the student of infectious disease has to assume that vi from a pragmatic angle; to the epidemiologist many viruses behave very much like organisms in general.' . . . 'A difficulty arises in regard the bacteriophages—or as they are coming to be called, bacterial viru —though Dr A. Felix adduced reasons for refusing to consider them being viruses at all. The trouble is to imagine, if the degradation hyp thesis is applicable, from what the phages could have been degrade Here Dr Burnet produced a new and fascinating theory: that they we the direct descendants of precellular stages in the evolution of livi forms; after the first bacteria were evolved, such primitive precellul creatures were at a great disadvantage and only those which adopte parasitic habit within the dominant bacteria were able to survive.' T article was not signed, but its author, who was Chairman at the 19 Symposium, is fortunately with us here and he may be able to confi the correctness of the report.

The reasons that prevented me from accepting the virus theory (theories) of phage and caused me to cling to Bordet's rival theory we the facts known about *Salmonella* phages. I shall state these facts ve briefly by means of a few slides. I know that some of those present ha seen these slides on previous occasions, but I need not apologize f that. Since the slides represent facts, not hypotheses, they have n changed in the course of a few years.

Table 1. *Salmonella Phages and Antigens*

So-called Typhoid Phages	Lytic action	Acting on	
		Antigens	*Salmonella*
Anti-O	Widely overlapping	IX XII_1 XII_2 XII_3 and many other O-antigen components	*typhi* *paratyphi-A, B, C* *typhi-murium* *enteritidis* and many others
Anti-Vi	Relatively specific	TVi	*typhi* *paratyphi-C* *ballerup* TVi-positive *coli*
Anti-H	Relatively specific Suppressing flagella	d	*typhi* *stanley*
Anti-R	Widely overlapping	R	All

Table 1 shows the well-known correlation between *Salmonella* phag and antigens, first established by Hadley (1925–6) and Burnet (192 1929) for O phages. Next followed the Vi phages described by Craigie

Brandon (1936), Sertic & Boulgakov (1936*a*) and Scholtens (1936). About the same time an anti-H phage was found by Sertic & Boulgakov (1936*b*). This is a difficult phage to work with and is little known, but it has been carefully investigated by Schiff & Bornstein (1940) and by Rakieten & Bornstein (1941). Its chief characteristic is to suppress the development of flagella. Finally, there is the anti-R phage which

Table 2. *Craigie's Adapted Typhoid Vi Phages*

Vi-type strains	Vi-phage preparations														
	A	B	C	D	E	F	G	H	J	K	L	M	N	O	T
A	CL	CL	CL	CL	CL	CL	CL	CL	CL	CL	CL	CL	CL	CL	CL
B	+ +	CL	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +
C	±	±	CL	±	±	±	±	±	±	±	±	±	±	±	±
D	—	—	—	CL	—	—	—	—	—	—	—	—	—	—	—
E	—	—	—	—	CL	—	—	—	—	—	—	—	—	—	—
F						CL									
G							CL								
H								CL							
J									CL						
K										CL					
L											CL				
M												CL			
N													CL		
O														CL	
T															CL

CL = Confluent lysis. — = No plaques. ±, + + = increasingly numerous plaques.

acts throughout the whole *Salmonella* group, but not beyond it. It is clear from this table that there is, in fact, no justification at all for calling any of these phages a 'typhoid phage' simply, without indicating the antigenic component to which it is related, because all these phages act not only on typhoid bacilli but also on other members of the *Salmonella* group.

If the different *Salmonella* phages are considered to be viruses of exogenous origin, parasitic on the bacterial cell, we have to assume that the distribution of the various O, Vi, H and R antigens in *Salmonella* has its counterpart in an exactly similar distribution of the corresponding specific receptors in *Salmonella* phages. To accept this hypothesis means putting too great a strain on the imagination.

Another set of observations is based on the work of Craigie and Yen (1938*a*, *b*) on adapted typhoid Vi phages.

Table 2 is an abridged version of the scheme now in use for the typing of typhoid bacilli. It shows only 15 different types, but at present the number of well-defined Vi-types and sub-types is 29. All the type-phages have been derived from a single strain of Vi-phage by the peculiar process of adaptation discovered by Craigie, and we must assume that the Vi-form of the typhoid bacillus contains at least 29 different determinant groups for Vi-phage. These cannot be detected by any of the customary serological methods, only by the corresponding receptors on the adapted bacteriophages. Again, if these phages are viruses of independent extrinsic origin, we have to postulate that nature has evolved two exactly corresponding sets of variants, or mutants, in two entirely distinct organisms, the typhoid bacillus and the virus-phage. I find it difficult to accept such a hypothesis.

Table 2 shows, what was known from Craigie's early work, that the first three types in the scheme (A, B and C) are susceptible to all the adapted phages, whereas all the other strains give strictly specific reactions. Yet, on prolonged subculture, or in a chronic carrier of long standing, every one of the specific Vi type may lose its initial resistance to some or all of the heterologous phages. For want of a better term, we call this process 'degradation'. It leads ultimately to a change from a specific Vi-type organism into Type A which is fully susceptible to all the adapted phages. The mechanism of this 'degradation' is quite obscure, but what is relevant for the purpose of the present discussion is the opposite feature, namely, the constancy of Vi-phage types. A striking example of this is illustrated in Table 3.

Three strains of *Salm. typhi* widely used in typhoid serology are shown in the table. Strain Ty2, isolated in Russia in 1918, is a highly virulent Vi+O+H form. Strain H 901, isolated at the same time and place, is a Vi-negative form of low virulence. Strain O 901, derived from H 901, is a pure O form, a permanent non-motile and Vi-negative variant. In 1936 Kauffmann succeeded in 'rejuvenating' the H 901 strain by mouse passage (Kauffmann, 1936) in the same way that Perry, Findlay & Bensted (1933) had accomplished the transformation of the avirulent Rawlings strain into one of high virulence. In this process the Vi-negative

Table 3. *Canstancy of Vi-phage Types*

Salm. typhi strains	Isolated	Antigens present in the strains	Vi-phage type
Ty2	1918 Cherson, Russia	H O Vi	E1
H 901	1918 Cherson, Russia	H O	.
O 901	1925	O	.
Rejuvenated H 901	Mouse passage Kauffmann 1936 Phage-typed Craigie & Yen 1938	H O Vi	E1

H 901 strain, after an interval of eighteen years, again developed its Vi antigen. Two years later Craigie & Yen (1938) typed the rejuvenated H 901 culture and the strain Ty2 and found that both belonged to the same Vi-phage type, namely, Type E1. At the time neither Kauffmann nor Craigie knew that the two strains had been isolated during the same outbreak in 1918. Naturally one could not fail to be impressed by the remarkable stability of this hereditary trait.

Similar relationships exist in *Salm. paratyphi B* and *A* and *Salm. typhi-murium* (Felix & Callow, 1943, 1951; Felix, 1951). There is here, therefore, a general principle and the virus theory of bacteriophage does not provide an explanation that can be accepted without undue credulity.

The observed facts can be explained with less strain on the imagination if we assume, according to Bordet's theory, that bacteriophages are endogenous products of the bacterial cell, specific self-reproducing units which form part of the genetic make-up of the bacterium.

Bordet's theory, according to which phage is derived from a precursor normally present in the bacterial cell (Bordet & Ciuca, 1920; Bordet, 1925, 1931), had its adherents until about ten years ago. The last notable exponents were Krueger (1936), Northrop (1938, 1940) and Wollmann (1925; Wollmann & Wollmann, 1932). Then the virologists got the upper hand. For the past few years I seem to have been the only surviving supporter of Bordet's original view (Felix, 1947, 1949) and I have been severely criticized. I am very glad therefore that Dr Lwoff has now produced a modernized version of the old theory (Lwoff & Gutmann, 1950; Lwoff, Siminovitch & Kjeldgaard, 1950).

One of the criticisms often levelled against this theory is that it revives the old argument about spontaneous generation reminiscent of Pasteur's period, and that it is therefore sterile from the beginning. For instance, the article in *Nature* from which I have quoted was in fact entitled 'Heterogenesis and the origin of viruses'. This criticism, I think, is entirely unjustified.

Since the virologists became the rulers in the sphere of bacteriophage work a very objectionable habit has become widespread, namely, to accept the findings in one particular and highly specialized field as valid generally throughout animal, plant and bacterial virology. A striking example is the case mentioned in Dr Luria's paper which refers to one of his own abandoned hypotheses for phage but which had meanwhile 'been seized upon eagerly by workers in other fields of virology and considered as "proved" for phage'. It is not unusual nowadays to read in a paper on rabies or vaccinia half a page or more dealing with 'the virus', which in fact refers to bacteriophage without stating this at all. Of course, some bacteriophage workers are also guilty of this disturbing habit.

Not only is it usually unjustified to draw analogies as far afield as from a bacteriophage to an animal virus but, more often than not, it is also wrong to assume that what is valid for one phage is also valid for others. In this way quite a number of 'rules' and 'laws' have been postulated which have no general validity at all. I may take as an example the so-called rule of the inverse ratio between particle-size and plaque-size, which is over-emphasized not only in text-books, but also in recent writings on the T series of *coli* phages. The fact is that the same phage when propagated under identical conditions on a number of strains of the same species may produce plaques varying in size by one-hundredfold or two-hundredfold, as I have shown with *Salmonella* phages (Felix, 1949). Other instances of conclusions drawn from a particular phage system and erroneously raised to the rank of a general rule or law refer to adsorption of phage by bacteria on which they cannot be propagated, as Craigie (1946) has shown with typhoid Vi phages; and to complete or incomplete neutralization of phage by anti-serum.

If I may be allowed to quote one more sentence from the report of the 1946 Meeting of this Society it will help me to conclude my remarks. 'Indeed, there was a general feeling throughout the discussion that a decision as to the origins of viruses should be taken on their merits for each group, animal, plant and bacterial viruses, and that a tendency for generalization to be too inclusive should be resisted.' It cannot be said that this advice was accepted during the intervening years.

The last point I wish to make is this. So far as bacteriophages are concerned it is known that their numbers are legion, as Dr Andrewes very aptly wrote in a recent article (Andrewes, 1951). If Bordet's theory of phage and of its role in bacterial variation is correct, it is not surprising that there are innumerable phages because there are innumerable enzyme-systems at work in bacteria. I have little doubt that refined methods of genetic analysis of bacterial variation, such as that of Lederberg, will reveal that the genetics of the bacteriophages form an integral part of the genetics of the corresponding bacteria. This will put bacteriophage in its legitimate place, in company with the many other lytic agents of bacteria that have been described by the Belgian workers (Gratia, Fredericq and Welsch), of which Dr Lwoff included only one in his tables.

I fully agree with Dr Andrewes' suggestion that the premature plan for nomenclature of bacteriophages be dropped (Andrewes, 1951). I believe that the final classification and nomenclature of bacteriophages will have to be accomplished by the chemist rather than by the virologist. Proteolytic enzymes already are classified by means of their inhibitors (Astrup & Alkjaersig, 1952). In the future it may perhaps become possible to identify phages by the antagonistic action of 'phage-analogues' in the same way that Fildes' school is using growth-factor analogues. In the present state of knowledge it would certainly cause less confusion if bacteriophage workers would avoid the misleading term 'virus', for which there is no accepted definition, and would continue to use the old and well-defined term bacteriophage.

REFERENCES

ANDREWES, C. H. (1951). Viruses and Linnaeus. *Acta path. microbiol. Scand.*, **28**, 211.

ASTRUP, T. & ALKJAERSIG, N. (1952). Classification of proteolytic enzymes by means of their inhibitors. *Nature*, **169**, 314.

BORDET, J. (1925). Le problème de l'autolyse microbienne transmissible ou du bactériophage. *Ann. Inst. Pasteur.* **39**, 717.

BORDET, J. (1931). The theories of the bacteriophage. *Proc. Roy. Soc. B.*, **107**, 398.

BORDET, J. & CIUCA, M. (1920). Le bactériophage de d'Hérelle, sa production et son interprétation. *C. R. Soc. Biol.*, **83**, 1293, 1296.

BURNET, F. M. (1927). Relationships between heat-stable agglutinogens and sensitivity to bacteriophage in Salmonella group. *Brit. J. Exp. Path.*, **8**, 121.

BURNET, F. M. (1929). ' Smooth-rough ' variation in bacteria in its relation to bacteriophage. *J. Path. Bact.*, **32**, 15.

CRAIGIE, J. (1946). The significance and applications of bacteriophage in bacteriological and virus research. *Bact. Rev.*, **10**, 73.

CRAIGIE, J. & BRANDON, K. F. (1936). Bacteriophage specific for O-resistant V form of *B. typhosus*. *J. Path. Bact.*, **43**, 233.

CRAIGIE, J. & YEN, C. H. (1938*a*). The demonstration of types of *B. typhosus* by means of preparations of Type II Vi phage. *Canad. Publ. Hlth. J.*, **29**, 448.

CRAIGIE, J. & YEN, C. H. (1938*b*). The demonstration of types of *B. typhosus* by means of preparations of Type II Vi phage. *Canad. Publ. Hlth. J.*, **29**, 484.

FELIX, A. (1947). The nature of bacteriophages. *Proc. 4th Int. Cong. Microbiol.*, p. 355. Copenhagen: Rosenkilde & Bagger.

FELIX, A. (1949). *The nature of the bacterial cell.* A symposium. p. 88. Oxford: Blackwell.

FELIX, A. (1951). Laboratory control of the enteric fevers. *Brit. Med. Bull.*, **7**, 153.

FELIX, A. & CALLOW, B. R. (1943). Typing of paratyphoid-B bacilli by means of Vi bacteriophage. *Brit. Med. J.* 2, 127.

FELIX, A. & CALLOW, B. R. (1951). Paratyphoid-B Vi-phage typing. *Lancet*, 2, 10.

HADLEY, P. (1925–6). Parallelism between serologic and bacteriophagic response in *B. typhosus* and certain avian paratyphoids. *Proc. Soc. Exp. Biol.*, **23**, 443.

KAUFFMANN, F. (1936). Untersuchungen über die Körperantigene in der Salmonella-Gruppe. *Z. Hyg. InfektKr.* **117**, 778.

KRUEGER, A. P. (1936). The nature of bacteriophage and its mode of action. *Physiol. Rev.*, **16**, 129.

LWOFF, A. & GUTMANN, A. (1950). Recherches sur un *Bacillus megatherium* lysogène. *Ann. Inst. Pasteur*, **78**, 711.

LWOFF, A., SIMINOVITCH, L. & KJELGAARD, N. (1950). Induction de la production de bactériophages chez une bactérie lysogène. *Ann. Inst. Pasteur*, **79**, 815.

NORTHROP, J. H. (1938). Concentration and purification of bacteriophage. *J. Gen. Physiol.*, **21**, 335.

NORTHROP, J. H. (1940). Increase in bacteriophage and gelatinase concentration in cultures of *Bacillus megatherium*. *J. Gen. Physiol.*, **23**, 59.

PERRY, H. M., FINDLAY, H. T. & BENSTED, H. J. (1933). Antityphoid inoculation; observations on variation of *Bact. typhosum in vivo*. *J. Roy. Army Med. Cps.*, **61**, 81.

RAKIETEN, M. L. & BORNSTEIN, S. (1941). Influence of certain bacteriophages on the H antigens of *Salmonella poona* and *E. typhi*. *Proc. Soc. Exp. Biol.*, **48**, 359.

SCHIFF, F. & BORNSTEIN, S. (1940). Hemolytic effect of typhoid cultures in combination with pure lines of bacteriophage. *J. Immunol.*, **39**, 361.

SCHOLTENS, R. T. (1936). Resistance developed against bacteriophage. *J. Hyg.*, **36**, 452.

SERTIC, V. & BOULGAKOV, N. A. (1936*a*). Sur la sensibilité des souches d'*Eberthella typhi* au bactériophage, en relation avec les caractères antigéniques. *C. R. Soc. Biol.*, **122**, 35.

SERTIC, V. & BOULGAKOV, N. A. (1936*b*). Bactériophages spécifiques pour des variétés bactériennes flagellées. *C. R. Soc. Biol.*, **123**, 887.

WOLLMANN, E. (1925). Recherches sur la bactériophagie (Phénomène de Twort-d'Hérelle). *Ann. Inst. Pasteur*, **39**, 789.

WOLLMANN, E. & WOLLMANN, E. (1932). Recherches sur le phénomène de Twort-d'Hérelle (bactériophagie). *Ann. Inst. Pasteur*, **49**, 41.

ASPECTS OF THE FORMATION OF INCOMPLETE VIRUS

SVEN GARD

Department of Virus Research, Caroline Institute, Stockholm

Luria (1950) describes the virus-host cell complex as an individual of a hybrid character, with the genetic structure of the virus and with the cytoplasm of the host. This description was intended for the system bacterium-bacteriophage but it seems to be applicable also to animal viruses. In the latter case, however, the genetic structure of the hybrid probably is more complex than in most phage-infected bacteria. In some cases the host genes are certainly not destroyed but still functioning. This is particularly obvious in virus-induced tumours. It seems not improbable that intermediates might occur as well, i.e. cases in which only part of the host genes are destroyed and replaced by virus genes. That Luria's description is not a mere play with words is brought out by the facts that the virus infected cell often shows characteristic morphological features as do the tumour cells, and a metabolism that differs distinctly from that of the non-infected cell, as shown by Ackerman (1951) and others.

The concept of the virus-host cell complex as a hybrid leads logically to the concept of the virus infection as a process of fertilization. There exist also some—maybe superficial—similarities that might excuse this extension of the analogy. Thus, mucopolysaccharides are very much in the centre of the interest as affinity determinants in both cases. A characteristic feature of fertilization is the impermeability of the cell membrane, induced by the intrusion of one single sperm cell. It is a well-known fact that only a small number of virus particles can be introduced into one and the same cell, although the spatial and 'receptorial' limits must be much wider. The most probable explanation is a radical change in the structure of the cell surface, induced in connexion with the entrance of the virus, i.e. a mechanism of the same principal nature as in fertilization. Phagocytosis, the only other process with which virus infection might be compared, seems to be of a completely different nature.

Irrespective of whether or not these ideas should be taken into serious consideration the first phase of the virus multiplication seems to be characterized by a reorganization of the cell to fit with its new function as a virus-producing unit. During this phase the destruction or alteration of the cell nucleus takes place, while no constituents showing any characters of the virus protein can as yet be detected.

The second phase might be termed the period of generation. The hitherto most valuable information about this phase seems to me to be obtained from the studies on influenza virus by Hoyle, and by Henle & Henle (1949) and Henle & Liu (1951). From their papers it would appear that a number of building-stones, identifiable in the final virus particle by their specific biological properties, are synthesized separately and independently, although sometimes simultaneously. This synthesis seems to proceed at a constant rate, another indication that no further production of enzymes takes place in the cell after intrusion of the virus (Monod & Wollman, 1947). A statement by Hoyle that the formation of group antigen first proceeds at a logarithmic rate is not borne out by his data.

The third and final phase is that of maturation. Whether or not this includes the production of any essential virus constituents is not clear. According to Rake & Blank (1950), studying the reproduction of molluscum contagiosum-virus, desoxyribonucleotides, considered to be of the most vital importance for the virus activity, should not appear until immediately before and during this stage. On the other hand, Hoyle seems to regard the maturation of influenza virus as a mere mechanical process, as a fitting of the building-stones or proviruses into the proper pattern, a welding together of the sub-units to final virus particles. It is possible that influenza virus is not a suitable model in this respect, the formation of the final particles apparently proceeding at the cell surface, whereas in most cases this must be supposed to take place in the interior of the cell. The question has to be left open for the time being. It can be stated definitely, however, that no active virus can be demonstrated in the cell until the stage of maturation has been reached.

The observation by von Magnus (1946, 1951*a*, *b*, *c*) that the cells under certain conditions release an incomplete influenza virus seems to me to be a finding of very great interest. Later, Hoyle (1948), Henle & Henle (1949), and Schlesinger (1950) described similar substances as constituents of infected cells which could be released by artificial means, and Bernkopf (1949) found that the production of incomplete virus is particularly striking in the chorio-allantoic membranes of de-embryonated eggs.

According to von Magnus multiple infection or rather saturation to full capacity of all the cells in the chorio-allantoic membrane is a prerequisite of the production of incomplete virus. The extent to which incomplete virus predominates over active virus in the final product is a function of the proportions between the two in the seed. It would seem, therefore, that incomplete virus induces the production of more incomplete virus. On the other hand, von Magnus supposes that no virus formation at all occurs unless there is at least some active virus present.

To obtain the largest possible yields of incomplete virus with a minimal content of active virus von Magnus employs serial passages of large inocula. Virus produced by diluted seed is called 'standard' virus, preparations obtained after inoculation of large doses in serial passages is called 'first, second, third, etc., passage' virus. This terminology will be used in the following.

The incomplete virus is characterized biologically by its haemagglutinating, enzymatic, and immunizing capacity but is apparently lacking in infectivity. In sedimentation studies Gard & von Magnus *et al.* (1946, 1952) found the incomplete virus to be less homogeneous than standard virus and to have a definitely lower sedimentation rate ($s_{20}=430$ to 650 S as against 745 S). It seems to differ in other physico-chemical respects as well, for instance with regard to viscosity.

It seems not improbable that production of incomplete virus is a much more common phenomenon than hitherto suspected. For instance, the appearance in virus infected plants of particulate matter resembling virus fragments a phenomenon particularly noticed by British authors, might belong in this category. The 'auto-interference' in *Salmonella* phage, recently described by Boyd (1951), shows a striking resemblance to von Magnus's observations. Finally, the production of immunity by antigenically insignificant amounts of live virus in resistant animals seems to indicate a production of virus antigen without simultaneous appearance of infectivity. Influenza virus offers unusually favourable conditions for the study of these phenomena, on account of the ease with which it can be assayed *in vitro*.

The observations by von Magnus and by Bernkopf suggest that metabolic deficiencies can be the cause of the formation of incomplete virus. On the other hand, the observation by Schlesinger that non-neurotropic strains of influenza virus, when inoculated intracerebrally, give rise to the formation of non-infectious virus haemagglutinin indicates that the disturbance of the virus synthesis can be localized to the genetic level as well. It seems to be a matter of taste, whether the cell or the virus is regarded as genetically deficient. From the fact that neurotropic strains of the same virus are able to propagate in the brain one would be inclined to stamp the non-neurotropic virus as deficient. On the other hand, as the same virus reproduces freely in the lung, the brain cells appear as equally deficient.

From these hints it follows that the study of the nature of the incomplete virus and the conditions under which it is formed might aid materially to a better understanding of the mechanism of virus formation. One can usually collect much more information about a certain reaction from studies of factors inhibiting it than from a direct study of the reaction itself. The more complex the process under study, the more true this should be.

As pointed out, the disturbance in the synthesis of the virus, leading to the liberation of an incomplete product, might be conceived to occur in the phase of reorganization or during the generation of virus protein, on account of shortage of one or more important metabolites. But it could also be the result of a disorganization of the 'assembling' mechanism during the phase of maturation. In the latter case, one would expect all the essential elements of the final virus particle to be present in the product, only haphazardly combined or maybe more probably only partially aggregated to form deficient particles. If Luria's (1947) concept of the virus infection is correct the phenomenon of 'multiplicity activation' would then seem a definite possibility. This problem seemed to be of sufficient importance to justify an experimental approach.

An answer to the question thus raised might be obtained by a study of the distribution of takes as a function of the infection dose in the range between 0 and 100 % takes. Before describing some experiments designed for this purpose, I must briefly touch upon the underlying principle and attempt to discuss its theory.

If a number of aliquot samples are taken from a suspension containing an average of x particles per sample volume, the chance distribution of particles will conform to a Poisson series. Thus the fraction of samples containing no particles will be e^{-x}, one particle $x.e^{-x}$, 2 particles $\frac{x^2}{2!}.e^{-x}$ ... n particles $\frac{x^n}{n!}.e^{-x}$. The fraction containing at least one particle will be

$$p = 1 - e^{-x}. \qquad (1)$$

Let the particles be virus particles and one single particle form the minimal infective dose. Then the formula (1) expresses the percentage of takes to be expected in a series of inoculations. The co-variation of p with x can be depicted diagrammatically as the so-called one-hit-curve (Fig. 1).

If at least two particles are necessary for infection the probability of takes will be

$$p = 1 - e^{-x} - x.e^{-x} \qquad (2)$$

the two-hit-curve. This expression is obtained on the assumption that any two particles will suffice to induce infection, irrespective of whether they are identical or not. The essential principle of the theory of multiplicity activation is the assumption that particles of different composition are able to complement each other to form infective units, whereas two identical, defective particles are not supposed to be able to induce infection. It is obvious, therefore, that the formula (2) cannot be applicable on this problem.

In trying to elucidate the phenomena to be expected in this case I will discuss only the simplest situation, i.e. the one in which only two kinds of incomplete virus particles appear. Let, as before, the average number of particles per unit volume be x, the fractions of incomplete particles a_1 and a_2, and the fraction of complete, active particles be b. We thus have

$$a_1+a_2+b = 1 \tag{3}$$

The fraction of samples containing no particles will be e^{-x}. Of the samples with one particle only those containing active virus will be infective. Thus a fraction corresponding to the expression $x(a_1+a_2).e^{-x}$ will be non-infective. Of the samples with 2 particles some will contain two identical incomplete particles and thus be non-infective. The expected combination of particles can be calculated by development of the expression $(a_1+a_2+b)^2$, and thus the fraction of non-infective samples should be $\frac{x^2}{2!}(a_1^2+a_2^2).e^{-x}$. With the same reasoning the total percentage of infective samples can be calculated to be

$$p = 1-e^{-x}-\left(a_1x+\frac{(a_1x)^2}{2!}+\frac{(a_1x)^3}{3!}+\ldots\right)e^{-x}-$$

$$-\left(a_2x+\frac{(a_2x)^2}{2!}+\frac{(a_2x)^3}{3!}+\ldots\right)e^{-x}$$

or

$$p = 1-e^{-(1-a_1)x}-e^{-(1-a_2)x}+e^{-x} \tag{4}$$

As a special case, for $b=0$, $a_1 = a_2 = 0{\cdot}5$, the formula (4) transforms into

$$p = \left(1-e^{-\frac{x}{2}}\right)^2 \tag{5}$$

The curves corresponding to (4) will assume different slopes depending upon the proportions of the different types of particles, as shown in Fig. 1. The presence of active virus will tend to decrease the slope and so will differences in the concentrations of the two kinds of incomplete virus. Thus, the curve corresponding to the formula (5) is hardly distinguishable from an ordinary two-hit-curve, whereas the proportions 90:10 of the incomplete viruses will give a curve that is practically identical with the one-hit-curve.

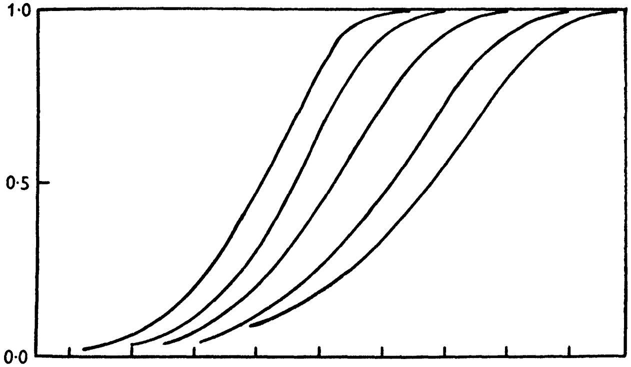

Fig. 1. Theoretical percentage of takes-dosage curves. Ordinate: $p =$ probability of take. Abscissa: $\log x$ (concentration of virus). The curves demonstrate from left to right $p = 1 - e^{-x} - x.e^{-x}$ (two-hit-curve); $p = 1 - e^{-(1-a_1)x} - e^{-(1-a_2)x} + e^{-x}$ (multiplicity activation) for $a_1 + a_2 = 1$ and $a_1/a_2 = 0{\cdot}5$, $0{\cdot}8$, and $0{\cdot}9$, respectively; $p = 1 - e^{-x}$ (one-hit-curve).

What has now been said applies also to curves of higher orders, i.e. those referring to cases where three or more particles are needed to form an infective unit. It is thus obvious that it is impossible, from the slope and shape of any experimental curve, to draw conclusions as to the actual number of particles necessary to induce infection. As a matter of fact the interpretation of a biological experiment is even more complicated, as two further factors tend to distort the picture. Firstly, a chance variation in the size of the inoculum will, of course, increase the standard deviation of x. Such variation is caused by errors of measurement, leakage from the site of inoculation, etc. Secondly, variations in the susceptibility of the test organisms are of very great importance. Differences in this respect can often be referred to the density of susceptible cells in an otherwise resistant tissue and need, therefore, not necessarily originate in constitutional differences of a heriditary nature. Both factors mentioned will act in the same direction, producing a percentage of takes-dosage curve of a flatter slope than the theoretical one-hit-curve. A steeper than normal course, on the other hand, can never result from such sources of error.

To conclude, an experimental percentage of takes-dosage curve of the theoretical or even flatter slope is not incompatible with the idea of multiplicity activation; a steeper than theoretical slope, on the other hand, is incompatible with the one-hit-theory and suggestive of multiplicity reactivation; no clue to the number of particles forming an infective unit can be obtained from experiments of this kind.

It must be emphasized that all multiple-hit-formulas hitherto discussed were calculated on the silent assumption that the entire inoculum be introduced into one and the same susceptible unit. Thus, x actually denotes the number of particles per cell. The formulas can always be applied to such cases where the fate of the individual cells can be observed, as in studies on bacteriophage or plaque-forming viruses in the chorio-allantoic membrane. It is not immediately clear, however, whether or not they are applicable on intra-allantoic influenza virus infection.

The number of lining cells in the allantoic cavity has been estimated at about 100 million. To obtain a positive result it is sufficient to infect one of these cells. Theoretically one single complete virus particle in the inoculum should, therefore, suffice to produce infection. With the aid of the formula (5) we can calculate the smallest number of incomplete particles that must be inoculated to produce infection of one single cell, i.e. the fraction 10^{-8} of the total number of cells. We have

$$10^{-8} = \left(1 - e^{\frac{-x}{2}}\right)^2$$
$$x = 0{\cdot}0002.$$

As pointed out above x denotes the number of particles per cell. The total number of particles that must be injected in the allantoic cavity is thus approximately 10^8 times greater or 20,000.

From the above reasoning we can conclude that, if we let x denote the number of particles per inoculum as is usual, the formula (1) can be applied unchanged to the infection with active virus but that for calculation of the probability of multiplicity activation (4) and (5) have to be transformed.

Let m be the number of cells exposed to the inoculum. The number of particles per cell will then be $\frac{x}{m}$. If this expression is substituted for x in (4) and (5), p gives the fraction of infected cells and $y = mp$ will be the average number of infected cells per egg. The distribution of infected cells in a series of inoculated eggs must be expected to be of the Poisson type and the percentage of takes can be calculated as follows:

$$p = 1 - e^{-y} \tag{4a}$$

where

$$y = m\left(1 - e^{-(1-a_1)\frac{x}{m}} - e^{-(1-a_2)\frac{x}{m}} + e^{-\frac{x}{m}}\right) \tag{4b}$$

For compution of the corresponding curves it is rational first to study the functions $\log(1 - e^{-x})$ and $\log(1 - e^{-x})^2$, shown in Fig. 2. Both functions, when plotted against $\log x$, assume a linear course in the range of small values of x. Thus, for values of $\frac{x}{m}$ below 10^{-1}, y will appear as an exponential function when plotted on the usual semilogarithmic scale.

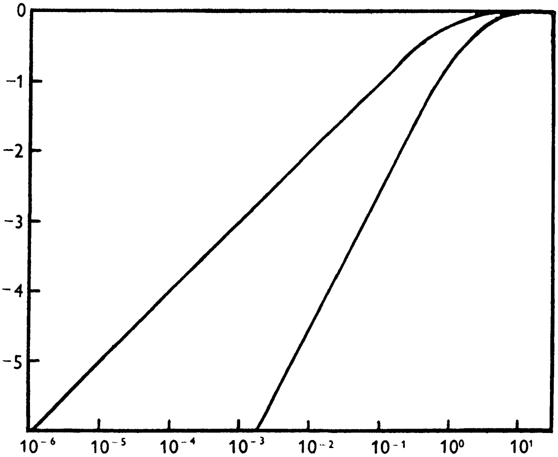

Fig. 2. Left: log $(1 - e^{-x})$, right: log$(1 - e^{-x})^2$ as functions of log x.

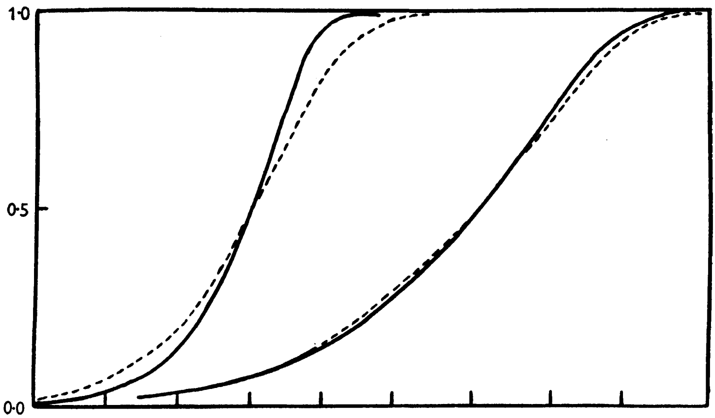

Fig. 3. Percentage of takes-dosage curves. Heavy lines: functions of the type (4a); left ${}^{a_1}/a_2 = 1$; right ${}^{a_1}/a_2 = \infty$. Fine broken line: functions (4) and (1), respectively.

Fig. 3 shows (4*a*) for $b = 0$ in the two extreme cases ${}^{a_1}/a_2 = 1$ and ${}^{a_1}/a_2 = \infty$. For comparison (4) and (1) are shown simultaneously.

With a mixture of active and incomplete virus the probability of takes will approximate to the sum of the functions (1) and (4*a*). It is obvious, therefore, that the effect of multiplicity reactivation will be noticeable only if the fraction of active virus is very small, 10^{-4} or less. However, this seems to be the case in a fair proportion of the preparations of 'third passage' virus.

As von Magnus has pointed out (1951) incomplete virus has the capacity of interfering with active virus. A mathematical treatment of this phenomenon will lead to an expression of the number of infected cells of the following principal form

$$y = m.\left(e^{-\frac{ax}{m}} - e^{-\frac{x}{m}}\right) \qquad (6)$$

where x is the total number of particles, a the fraction of interfering particles, and m the number of cells exposed. The corresponding curve is shown in Fig. 4. For small x values p increases with x, then passes through a maximum at $x = \frac{m}{1-a} ln\frac{1}{a}$, and again decreases. The agreement with experimental results in mice, published by von Magnus, is good.

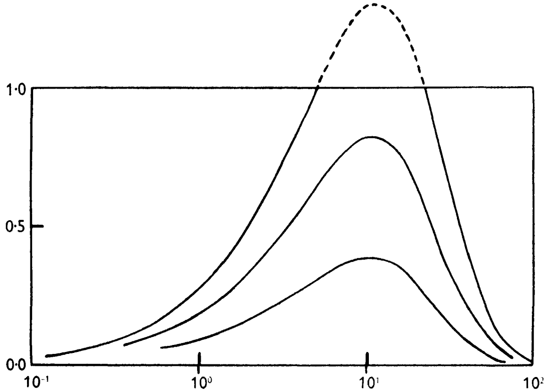

Fig. 4. 'Auto-interference' curves: $y = m\left(e^{-\frac{ax}{m}} - e^{\frac{x}{m}}\right)$; $m = 10$; $a = 0{\cdot}7, 0{\cdot}8$, and $0{\cdot}9$, respectively. The broken line denotes the range with an average of more than one infected cell per test organism.

So long as no pure incomplete virus can be obtained without even traces of active virus a very complex picture has to be expected. There must occur simultaneously single hit infections, interference phenomena, and possibly multiplicity activation. On our present level of knowledge it would be futile to try to construct a composite percentage of takes-dosage curve. There is but one thing to do: collect empirical data.

The experiments to be described were performed in co-operation with Dr Arne Svedmyr. The specimens used in the tests were a preparation of PR8 influenza virus, the Stockholm substrain, known to give standard passage virus in which no traces of incomplete virus are demonstrable on sedimentation analysis, and a third passage of the same strain. The difference in infectivity titre between the two specimens amounted to about 3·5 log units.

With each preparation a total of 7 titrations in two-fold dilutions in groups of 40 to 80 eleven-day eggs were performed, and the results recorded after 48 or 72 hours.

The mean percentages of takes through all 7 experiments are shown in Fig. 5, in which the 50% point, calculated on the basis of the three central points of the titrations curves, forms the reference point.

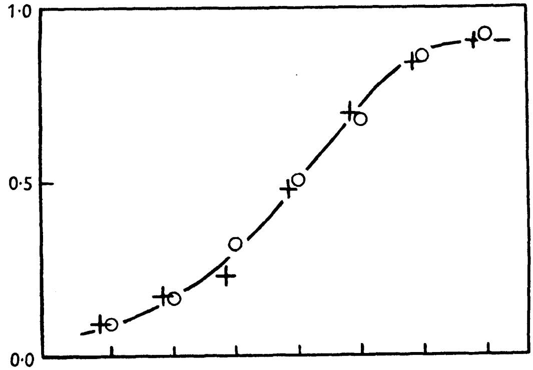

Fig. 5. Mean percentages of takes from 7 titrations of 'standard' and 'third passage' PR8 influenza virus. The curve corresponds to the function $p = 0\cdot9(1 - e^{-x})$.
0 = standard virus
+ = third passage virus.

Concentrating first on the standard virus we find that the slope of the titration curve is flatter than that of the theoretical one-hit-curve. It is also remarkable that the curve levels off already at about 90%. This might happen if by technical faults a certain proportion of the inocula were injected extra-allantoically, if a considerable leakage occurred after inoculation, or if a certain proportion of the eggs were more resistant than the majority. Whatever the reasons might be, the experiments were planned to give an even distribution of errors of this nature over the whole material, and the standard and third passage titrations should be directly comparable. The fit to a curve giving 90% of the theoretical p values is good.

There seems to be no appreciable difference between standard and third passage when the whole range of concentrations is considered. If, however, only the central part of the curve is analysed—where the slope is maximum—a certain difference might be discernible. Table 1 shows an analysis of this kind. In this table for each experiment the attack rate of the dilution giving approximately 70% takes has been compared

Table 1

	Exp. No.	% takes		Difference $(x-y)$	
		higher conc. (x)	lower conc. (y)		
standard	1	62·5	45·0	17·5	Mean diff. 35.5
	2	67·4	36·0	31·4	
	3	68·0	28·3	39·7	
	4	68·0	22·0	46·0	
	5	70·8	28·0	42·8	
	6	73·9	36·7	37·2	
	7	62·0	28·2	33·8	
third passage	1	63·3	16·3	47·0	Mean diff. 46.6
	2	69·4	27·1	42·3	
	3	69·4	15·9	53·5	
	4	68·0	22·5	45·5	
	5	73·3	22·9	50·4	
	6	77·1	34·0	43·1	
	7	64·1	19·5	44·6	
Difference third passage—standard					11·1 ± 4·11

with that of a four times higher dilution. The mean difference in attack rate between the two points is 35·5 and 46·6% for standard and third passage, respectively. Thus, standard and third passage differ in this respect by 11·1%, a difference that would occur by chance not more than 1 to 2 times in 100 tests.

A complete co-variation analysis of the material pertaining to the three central points gives coefficients of regression of 0·181 and 0·231 respectively. In this case the significance of the difference is slightly less, as it would happen between 2 and 5 times out of 100.

It is obvious that the present experiments do not permit a definitive answer to our question. As pointed out above, experiments of this type can hardly ever disprove the hypothesis of multiplicity reactivation, even when the results are completely negative. In our case the outcome is—if anything—suggestive of a positive result, although it would be hazardous to draw any conclusions. Further experiments along the same line must be regarded as highly justified. It then is of importance to try to obtain a preparation of incomplete virus better suited for the purpose. As already mentioned, admixture of active virus tends to make the effect of multiplicity activation less noticeable or cover it completely. Our present preparation of third passage virus with an infectivity titre only 3·5 log units less than that of the active virus can probably not be regarded as quite satisfactory.

It might seem unwise to present our material in its present preliminary state. However, the main reason for this paper was not to describe the experiments as such but to raise a discussion of the theory of multiplicity activation, a problem of the utmost importance for the understanding of the mechanisms of virus infection and virus multiplication.

REFERENCES

ACKERMAN, W. W. (1951). The role of l-methionine in Virus Propagation. *J. Exp. Med.*, **93**, 337.

BERNKOPF, H. (1949). Cultivation of influenza virus in the chorioallantoic membrane of deembryonated eggs. *Proc. Soc. Exp. Biol. Med.*, **72**, 680.

BOYD, J. S. K. (1951). Observations on the relationship of symbiotic and lytic bacteriophage. *J. Path. Bact.*, **63**, 445.

GARD, S. & VON MAGNUS, P. (1946). Studies on interference in experimental influenza. II. Purification and centrifugation experiments. *Ark. Kemi Min. Geol.*, **24 B**, No. 8.

GARD, S., VON MAGNUS, P., SVEDMYR, A. & BIRCH-ANDERSEN, A. (1952). Studies on the sedimentation of influenza virus. *Arch. Virusforsch.* (in press).

HENLE, W. & HENLE, G. (1949). Studies on host-virus interactions in the chick embryo-influenza virus system. III. Development of infectivity, haemagglutination, and complement fixation activities during the first infectious cycle. *J. Exp. Med.*, **90**, 23.

HENLE, W. & LIU, O. C. (1951). Studies on host-virus interactions in the chick embryo-influenza virus system. VI. Evidence for multiplicity reactivation of inactivated virus. *J. Exp. Med.*, **94**, 305.

HOYLE, L. (1948). The growth cycle of influenza virus A. A study of the relations between virus, soluble antigen and host cell in fertile eggs inoculated with influenza virus. *Brit. J. Exp. Path.*, **23**, 390.

HOYLE, L. (1950). The multiplication of influenza viruses in the fertile egg. *J. Hyg.* **48**, 277.

LURIA, S. E. (1947). Reactivation of irradiated bacteriophage by transfer of self-reproducing units. *Proc. Nat. Acad. Sci.*, **33**, 253.

LURIA, S. E. (1950). Bacteriophage. An essay on virus reproduction. *Viruses, 1950*, p. 7. California Institute of Technology, Pasadena, California.

VON MAGNUS, P. (1946). Studies on interference in experimental influenza. I. Biological observations. *Ark. Kemi Min. Geol.*, **24 B**, No. 7.

VON MAGNUS, P. (1951*a*). Propagation of the PR8 strain of influenza A virus in chick embryos. I. The influence of various experimental conditions on virus multiplication. *Acta path. microbiol. Scand.*, **28**, 250.

VON MAGNUS, P. (1951*b*). Propagation of the PR8 strain of influenza A virus in chick embryos. II. The formation of ' incomplete ' virus following inoculation of large doses of seed virus. *Acta path. microbiol. Scand.*, **28**, 278.

VON MAGNUS, P. (1951*c*). Propagation of the PR8 strain of influenza A virus in chick embryos. III. Properties of the incomplete virus produced in serial passages of undiluted virus. *Acta path. microbiol. Scand.*, **29**, 157.

MONOD, J. & WOLLMAN, E. (1947). L'inhibition de la croissance et de l'adaptation enzymatique chez les bactéries infectées par le bactériophage. *Ann. Inst. Pasteur*, **73**, 937.

RAKE, G. & BLANK, H. (1950). The relationship of host and virus in molluscum contagiosum. *J. Invest. Dermatol.*, **15**, 81.

SCHLESINGER, R. W. (1950). Incomplete growth cycle of influenza virus in mouse brain. *Proc. Soc. Exp. Biol. Med.*, **74**, 541.

DISCUSSION

L. HOYLE: How long a period elapsed between collection of allantoic fluid from the eggs used and the performance of the centrifugation experiments? There is reason to believe that particles of virus are more hydrated immediately on leaving the cells than is the case after a period of storage and this may affect measurements of particle size.

S. GARD: Some three days. This would be long enough to allow stabilization of the particles.

L. HOYLE: What is the latest estimate of the particle size of the incomplete virus?

S. GARD: For certain reasons (differences in specific volume) the sedimentation constants are not a reliable measure of the relative particle sizes. The results of sedimentation studies indicate, however, a considerable heterogeneity of the incomplete virus, which is further borne out by electron microscope observations showing the presence of particles of greatly varying size, from 120 mμ downwards.

C. H. ANDREWES: Dr Gard has said that presence of much active virus shifts production in favour of active virus and similarly incomplete virus pushes things the other way. Yet essentially excessive doses of active virus seem to lead to incomplete virus production. Again when active virus is at a minimum after several passes and incomplete virus is dominant, there seems to be a shift back again to active virus. There seems a discrepancy here.

S. GARD: Generally speaking, the production of incomplete virus requires fulfilment of two conditions: (*a*) multiple infection, (*b*) presence in the inoculum of some complete virus. The proportions of the two variants in the yield is dependent upon their proportions in the inoculum. I will not try to explain this empirically found fact. The reason for a shift towards larger yields of active virus after 3 or 4 passages is to be found in the fact that the total yield decreases and consequently also the multiplicity of infection in the subsequent passages.

D. J. BAUER: It would be interesting to know what happens to the second component when more than three passages are carried out. If the curves continue the same trend the paradoxical situation will arise in which a haemagglutin is being propagated which is not infectious.

S. GARD: According to von Magnus the total yield of virus protein is somewhat smaller when the proportion of incomplete virus reaches maximum. In a subsequent passage the multiplicity of infection will thus be lower and the result is a product with more complete virus. Experiments with artificially concentrated seed virus have not yet been made.

F. C. BAWDEN: We have not yet achieved the position postulated by Dr Bauer, for a wholly non-infective antigen would not be perpetuated, but we have approximated to it with broad bean mottle virus (Bawden, Chaudhuri & Kassanis, *Ann. Appl. Biol.*, 1951, **38,** 774). With most plant viruses there is a factor of about 1/1000 between the precipitin titre and infectivity of extracts from infected plants. For instance, with tobacco mosaic virus the precipitation- and infection-end points are about 1/1,000, and 1/1,000,000, and with potato virus Y 1/20 and 1/10,000 respectively. With broad bean mottle virus, by contrast, the precipitation- and infection-end points are almost the same. Infected leaves give extracts containing as much as 2 g. of anomalous nucleoprotein per litre, but most of this seems to be non-infective.

THE MULTIPLICATION OF THE INFLUENZA VIRUS CONSIDERED IN RELATION TO THE GENERAL PROBLEM OF BIOLOGICAL MULTIPLICATION

L. HOYLE

Public Health Laboratory, Northampton

It is very probable that all biological multiplication is fundamentally similar in nature, and that a complete understanding of the multiplication of a simple living agent would be of the greatest help in understanding the behaviour of more complex forms. It is for this reason that a study of virus multiplication is of more than ordinary importance and interest.

INTRACELLULAR MULTIPLICATION OF THE INFLUENZA VIRUS

The following are the essential facts which have been observed in recent years in studies of the multiplication of influenza virus in the allantoic sac of the fertile egg (Hoyle, 1948, 1950, 1952; Henle, 1949*a*, *b*; Henle & Henle, 1949; Burnet, 1951*a*, *b*; Burnet & Lind, 1951*a*, *b*).

The influenza virus elementary body is equipped with a specific property—the red cell agglutinin—which has an affinity for mucoprotein receptors present in the wall of susceptible host cells (Hirst, 1942, 1948). The first stage in infection is a union between the agglutinin of the elementary body and the cell receptors. The virus also possesses an enzyme which is able to destroy the cell mucoprotein, but infection is not dependent on the action of this enzyme. The elementary body appears to be actively taken up by the cell itself (Burnet, 1948; Fazekas de St Groth, 1948). On entry into the cell the elementary body disappears and for a period of about 2 hours no evidence of the presence of virus in the cell can be obtained. Extracts of the chorio-allantoic membrane at this stage do not agglutinate red cells and have an infectivity titre which is so low that it probably represents merely stray virus which has not entered the cells at all. The extracts also contain no demonstrable complement fixing antigen, though this finding is of little significance since the complement fixation test is barely sensitive enough to detect the amount of virus which can be induced to enter the cells.

The first evidence of growth of the virus is the appearance at 2 hours of complement fixing soluble antigen in the cells. The antigen increases rapidly between 2 and 4 hours and ultimately attains an amount about 100 times that present in the original inoculum. The antigen is not infective and does not agglutinate red cells. Chemical studies (Hoyle, 1952) indicate that it is probably a ribonucleoprotein. The particle size of the antigen is smaller than that of the infective virus.

Extracts of infected chorio-allantoic membrane develop the ability to agglutinate red cells between 3 and 4 hours after inoculation. The appearance of the agglutinin is rather sudden, but the amount which appears does not become greater than that present in the original inoculum until after about 4 hours. The appearance of agglutinin coincides with the appearance of a strain-specific complement fixing antigen which is distinct from the non-specific soluble antigen. Both these agents, agglutinin and specific antigen, are adsorbed by red blood cells and it seems probable that they are identical. The agglutinin is not infective, but possesses enzymic properties similar to those of the elementary body, uniting with and destroying the mucoprotein receptors of red cells. Under certain circumstances non-infective agglutinin may appear in the allantoic fluid and has been found to have a particle diameter about half that of the elementary body (Gard & von Magnus, 1946; von Magnus, 1951*a*, *b*, *c*).

Infective virus does not appear until between 5 and 6 hours, and is immediately excreted into the allantoic fluid. The observations suggest that infectivity is acquired as a result of excretion from the cells. From 6 hours onward there is a rapid rise in the infectivity and agglutinin titre of the allantoic fluid. If the fluid is examined in the dark background microscope a variety of particles are seen which are not found in normal allantoic fluid. These include numerous elementary bodies of particle size 1,000–1,500 A.U., filaments of diameter 1,250 A.U., and small particles of sizes ranging from 120–600 A.U. In addition large spherical globules of from 2,000–50,000 A.U. may be seen. All these particles may be adsorbed by red cells and subsequently eluted. Examination of the cells of the chorio-allantoic membrane in the dark background microscope at the time when the agglutinin titre of the allantoic fluid is rising shows that spherical and tubular protrusions appear from the margins of the cell, and may break away from the cells. The tubular protrusions often segment into bead-like chains. On breaking away from the cells all types of protrusion tend to shrink, and it seems probable that the elementary bodies and filaments present in infected allantoic fluid are derived from the cells in this way. Very similar appearances are seen in electron microscope preparations (Eddy & Wyckoff, 1950; Murphy, Karzon & Bang, 1950; Wyckoff, 1951), the fragmentation of filaments into bead-like chains being well seen, but the larger types of cell protrusion are not seen in electron microscope preparations as they are probably too delicate to withstand drying and fixation.

The whole process suggests a disturbance in the balance between the internal osmotic pressure in the cell and the strength of the cell membrane, possibly a result of the enzymic action of the virus on the cell wall. Very similar protrusions can be produced from normal cells under conditions in which the osmotic balance becomes disturbed.

STRUCTURE OF THE VIRUS ELEMENTARY BODY

If a preparation of elementary bodies made from infected allantoic fluid by adsorption and elution from red cells is shaken with ether the elementary bodies and filamentous forms disintegrate. A lipid can be recovered from the ether which has serological characters suggesting that it is derived from the host cell. The aqueous phase contains only small particles of two distinct types, one a complement fixing particle apparently identical with soluble antigen, and a second particle with red cell agglutinating properties. Both particles have a size of 120 A.U. but aggregates of larger size are also found of which one of 500 A.U. diameter is common (Hoyle, Reed & Astbury, 1952). The complement fixing soluble antigen particle is apparently a ribonucleoprotein. It has the general properties of a protein; it forms an insoluble complex with lanthanum salts; it is partially inactivated by crystalline ribonuclease and more completely inactivated by trypsin. If it is treated with alcohol at room temperature a serologically active fraction, probably nucleic acid, can be recovered from the denatured precipitate. The red cell agglutinating particle has the chemical properties of a protein and shows enzymic activity, destroying the mucoprotein receptors of the red cell. The serological properties of the agglutinin are peculiar. Unlike the intact elementary body and the soluble antigen it does not react in complement fixation tests if short fixation is used, but if the fixation time is prolonged it gives complement fixation both with strain specific sera and also with group sera reacting only with soluble antigen. (Hoyle, 1952).

Electron microscope pictures of intact elementary bodies show many particles which are not truly spherical but show an appearance suggesting a hexagon with rounded corners. The first stage in ether disintegration appears to be a collapse of a surrounding membrane, the body becomes flat and swollen and is clearly seen to contain many particles of smaller size. The hexagonal form may be retained, the body appearing as a rounded flat hexagon. Further treatment with ether results in total disintegration with the release of numerous small particles. The elementary body thus seems to consist of a packet of smaller units of two types—soluble antigen and agglutinin—enclosed in a lipid membrane, and the hexagonal form probably results from hexagonal close packing of the smaller units.

Any satisfactory theory of virus multiplication must fit in with the above facts and in addition must offer some explanation of the phenomenon of adaptation, the existence of different variants of the same virus, the artificial production of variants in the laboratory, and the phenomenon of interference.

THEORIES OF MULTIPLICATION

Many workers with animal viruses have believed that these resemble bacteria and that their multiplication in the cells was similar to that of bacteria in a culture medium. The phenomena described above are quite incompatible with any such view. If it were true then in the course of intracellular multiplication the various properties of the virus should become detectable in the following order, dependent on the relative sensitiveness of the tests used: Virus should first be demonstrable by infectivity tests, then by red cell agglutination, and finally by complement fixation. In fact the observed result is the exact opposite, increase in complement fixing antigen is detectable at 2 hours, red cell agglutinin becomes detectable at 3 hours and increases above the original level at 4 hours, while the infectivity titre does not rise until 5–6 hours. Further no virus can be detected in the cell by any method in the first 2 hours. The original inoculum appears to be destroyed.

But a more serious criticism can be made. Bacteria do not multiply by fission. The statement that a bacterium on introduction into a culture medium first enlarges in size and then divides is true, but offers no explanation of the phenomenon of biological multiplication. The problem is the increase in size which precedes fission. Fission has nothing to do with multiplication but is concerned with survival. By dividing an organism doubles its chances of survival, but multiplication in the sense of increase in substance can occur without fission.

It is worth while to consider what must be happening when a bacterium increases in size on being introduced into a culture medium. The organism contains many kinds of molecules of much larger size and complexity than those available in the medium. All these molecules increase in number. Most of the large molecules are produced by the action of enzymes, but the enzymes are themselves increased and must be produced from some other precursor or gene. In the end there must be some unit or units which are self-replicating and it seems probable that these fundamental units are nucleoproteins. The multiplication of a bacterium must therefore be divisible into three stages, nucleoprotein replication, enzyme production, and enzyme action. This is precisely what occurs in the intracellular growth of the influenza virus. The nucleoprotein soluble antigen increases first, then enzymically active agglutinin appears, and finally the virus escapes from the cell as a result of the action of the enzyme on the cell wall. The suggestion that viruses or bacteria multiply by fission is merely an evasion of the fundamental issues.

Two theories have so far been advanced which offer a reasonably satisfying explanation of the observed facts, that of the writer (Hoyle, 1948, 1950) and that of Burnet & Lind (1951).

Hoyle's hypothesis

This concept may be briefly described as follows. The elementary body consists of a packet of smaller units, soluble antigen and agglutinin, enclosed in a lipid envelope derived from the wall of the host cell. The agglutinin is responsible for the initial union with the cell. On entry into the cell the lipid envelope fuses with the wall of the host cell, the elementary body is disintegrated and the smaller units enter the cell. The soluble antigen is a self-replicating ribonucleoprotein which multiplies in the cell. At a later stage agglutinin is formed from the soluble antigen, the particular type of agglutinin being conditioned by the presence of agglutinin from the original inoculum which acts as a pattern. The agglutinin and soluble antigen tend to aggregate and the complex possesses enzymic activity and is able to destroy the mucoprotein of the cell wall. This results in a mechanical weakening of the cell wall and protrusions develop which contain aggregating virus protein. The protrusions break away from the cell and ultimately form elementary bodies as a result of loss of water and continuing internal aggregation of the virus units.

Hypothesis of Burnet and Lind

This was derived from a study of variation in influenza viruses and is very similar to the concepts advanced in recent years by workers with bacteriophage (Luria, 1950). Burnet & Lind found that if cells are infected with two strains of virus with different biological properties, under suitable conditions a new strain can be isolated with properties partly derived from one of the original strains and partly from the other. They regard the virus as a complex of a number of different smaller units each of which possesses self-replicating powers and which they regard as genetic units. On entry into a cell the body disintegrates, the various genetic units replicate independently, and ultimately infective virus is formed by a process of aggregation of the smaller units.

There is clearly considerable similarity between the two hypotheses, but each presents special advantages and difficulties.

The Problem of Self-Replication

Both theories involve the concept of the existence of large macromolecular or genetic units with self-replicating powers. It is difficult to understand how such self-replication can occur. Viruses do not contain the enzymes necessary for protein synthesis (Bauer, 1949), and there is no reason to suppose that a gene is able to synthesize itself from nutrient materials entering the cell. The cell must contain a system capable of synthesizing nucleoprotein but as there is no reason to suppose the existence of a special synthesizing enzyme for each individual protein or nucleic acid the system must be non-specific in that it is capable of producing any nucleoprotein. The self-replicating unit must enter the system at some stage and direct the synthesis so as to produce a replica of itself. Two methods have been suggested. According to the first the molecule increases by the addition of smaller units from its environment in a series of layers, the molecular structure of the underlying layer determining that of the superimposed one. The process would be similar to crystallization (Troland, 1917; Stanley, 1938). Such an idea seems to involve a progressive increase in size of the macromolecule, and it is therefore necessary to suppose that at a later stage the enlarged form breaks into smaller units. However the appearance of inclusion bodies in cells in many virus diseases, and in some cases the appearance of intracellular crystals might suggest some such process. The second idea is that of the intervention of a negative template (Gulick, 1938; Langmuir & Schaefer, 1938; Pauling, 1940; Darlington, 1944). The original macromolecule creates from its environment a molecular grouping which is its own opposite and this template in turn reproduces the original molecule. A preliminary unfolding of the nucleoprotein chain seems to be necessary. The template would have to have a structure as large and as complicated as the original macromolecule and it seems evident that the template would itself be a nucleoprotein. If this were so it would itself be a replicating unit, and one might consider the possibility that nucleoprotein molecules are composed of two equal and opposite halves, each half acting as a template for the synthesis of the other.

It is also possible that the actual direction of nucleoprotein synthesis is not due to the intact nucleoprotein molecule itself but to some smaller unit which results from its disintegration.

Whatever the mechanism may be the existence of large nucleoprotein units with genetic continuity cannot be doubted.

Interference between replicating molecules

The hypothesis of Burnet & Lind involves the simultaneous multiplication in the cell of a number of different genetic units. This raises the question as to whether it is possible for a number of different replicating units to exist in the same system at the same time. If all such units are nucleoproteins they will be built of the same basic materials. The rate of replication will be conditioned by the supply of basic materials. The multiplication of soluble antigen in cells infected with influenza virus is dependent on the adequate supply of nutrient material from outside. Bacteriophage synthesis is similarly dependent on nutrient material entering the cell. There is also evidence that virus synthesis in cells is carried out at the expense of normal protein synthesis.

If two different units are replicating in a cell at the same time, as soon as the amount present becomes so large that the supply of nutrients becomes inadequate there will be a competition for the available material, and whichever replicating unit is present in largest amount will be more likely to secure the nutrient and will therefore increase relative to the other. This process must ultimately result in one unit overwhelming the other. It is therefore possible that only one replicating unit can exist in a cell at one time. This might explain the phenomenon of interference between viruses. A cell infected with influenza virus A will not support the growth of virus B introduced later. It also suggests that a certain minimal dose of virus protein must be introduced into a cell in order to overcome the normal protein synthesis. This might explain why the precursor of Gard & von Magnus, a particle of essentially the same structure as the elementary body but only about one eighth of its volume, is apparently non-infective.

There is however one condition in which this reasoning would not apply. If a number of replicating units A B C D are responsible for the production of a number of enzymes a b c d, and the presence of all these enzymes is necessary for the synthesis of any of the units A B C D, then these units will increase in parallel, a state of symbiosis will exist in which the replication of one unit is dependent on the simultaneous replication of the others. Such interdependent units might well form an aggregate or complex in the cell, and it is possible that some of the larger particles present in cell cytoplasm are aggregates of such interdependent units.

Fundamental role of the soluble antigen

The writer believes that all strains of influenza virus A contain a fundamental replicating nucleoprotein, the soluble antigen, which is the same in all strains of virus A and differentiates virus A from virus B. Burnet & Lind would regard the soluble antigen as only one of a number of equally important replicating units and if their view is adopted there is no essential difference between virus A and virus B and it should be possible by the methods used by Burnet & Lind to produce a hybrid strain in which the soluble antigen of virus A is combined with the agglutinin of virus B. This would be much less likely to occur if the writer's hypothesis is correct. The experiment has not yet been tried, but it is perhaps significant that no such hybrid has ever been isolated in nature. The possession of soluble antigen of A type seems to involve the possession of certain types of agglutinin associated with strains of A type and is not compatible with the possession of agglutinins of B type.

In growth cycle experiments the multiplication of soluble antigen precedes the appearance of all other activities, suggesting that this unit plays a fundamental role. On the view of Burnet & Lind multiplication of soluble antigen and agglutinin should proceed simultaneously. It has been suggested by Liu & Henle (1951) that this is really true but that the agglutinin is not detected in the early stages because it combines with an inhibitor present in the chorio-allantoic membrane. They find that if membrane extracts are prepared with a period of treatment with the receptor destroying enzyme of *V. cholerae*, agglutinin is released from combination with inhibitors and is then detected at the same time as soluble antigen. The writer has studied this question with some care and is unable to agree with the interpretation. If in the preparation of membrane extracts the suspension is incubated for some hours as in the method of Hoyle (1948) the agglutinin present is able to destroy the inhibitor, and in this case there is little increase in yield if receptor destroying enzyme is used. The time of detection of soluble antigen and agglutinin is dependent on the sensitiveness of the method of detection. If the two agents were multiplying simultaneously the agglutinin should be detected first in view of the greater sensitiveness of the agglutination test as compared with complement fixation. This result never occurs. If the yield of agglutinin and soluble antigen is measured in terms of the amounts of these agents present in the virus originally inoculated it is found that increase of soluble antigen precedes increase of agglutinin by about 2 hours. Soluble antigen production also precedes agglutinin production in the lungs of infected mice (Fazekas de St Groth & Donnelly, 1950).

Northrop, Kunitz & Herriot (1948) have suggested a mechanism of protein and enzyme synthesis in cells which is of interest in this connection. They suggest that cells first synthesize a species—specific proteinogen, the energy for this synthesis being obtained by a transfer by means of coupled reactions of the energy obtained by breakdown of nutrient materials. The proteinogen itself directs the synthesis to reproduce its own structure. It is further suggested that the proteinogen contains nucleic acid and has characters resembling a denatured protein. From this proteinogen the various specific native proteins of the cell are produced by a catalytic reaction. This second stage would not require energy and could take place anywhere in the cell. The production of an enzyme from its precursor is cited as an example. The soluble antigen appears to have the characters of such a proteinogen and might well act as a precursor of the enzymically active agglutinin. The writer suggested (Hoyle, 1950) that most of the agglutinin was synthesized during the attack of the virus on the cell wall in the later stages of intracellular growth. It may be objected that this synthesis required energy, but if the agglutinin is produced from soluble antigen according to the scheme of Northrop, Kunitz & Herriot this difficulty disappears since the conversion of a denatured protein into a native protein does not require energy.

Serology of the influenza viruses

Influenza viruses can be divided into two quite clear-cut groups, A and B by means of the complement fixation reaction given by their soluble antigens. By the haemagglutination test each group can be subdivided into different strain types, but these are not sharply defined, although one particular type of haemagglutinin may be dominant in a given strain small amounts of other haemagglutinins are usually present. There is however no relation at all between the haemagglutinins of A and B viruses. If as supposed by the writer the complement fixing soluble antigen is a fundamental replicating unit while the various haemagglutinins represent different enzymes which can be produced from it the serological results are completely explained. On the theory of Burnet & Lind the results are much less easily understood and could certainly not have been predicted.

Problem of the fate of the original inoculum

The disappearance of the original inoculum is one of the most difficult problems of virus multiplication. The chorio-allantoic membrane will take up between 500 and 1,000 agglutinin units of virus. But one hour after inoculation no agglutinin can be demonstrated in membrane extracts, even with the use of receptor destroying enzyme. Nor can virus be demonstrated by complement fixation tests, even if prolonged fixation is used to increase the sensitiveness of the test. The virus originally entering the cells is not merely disintegrated into agglutinating and complement fixing units, it seems to entirely disappear. It might be that on entry into the cell the virus became so firmly linked to some cell constituent that it could not afterwards be demonstrated, but such an effect should be equally well demonstrable under *in vitro* conditions. Some destruction of virus does occur under *in vitro* conditions, and this effect is produced by some constituent of the cell stroma. If a frozen and thawed suspension of chorio-allantoic membrane is centrifuged the supernatant fluid does not destroy virus but the resuspended sediment does. However the destruction demonstrable *in vitro* is very much less complete than in the living egg, suggesting that the effect is not solely due to adsorption to some cell constituent but is an active process involving energy changes.

It has sometimes been suggested that the union of virus with a cell is similar to the fertilization of an ovum, in that only one particle can enter the cell and that this produces some change in the cell surface which ensures the destruction of particles arriving later. But there is no evidence of the survival of even one particle per cell. The allantoic sac is lined by some 100–200 million cells so that even if only one particle entered each cell, virus should be readily demonstrable in membrane extracts. There is however a limit to the number of particles which will unite with the cells, and this may possibly be due to destruction of receptors on the cell surface.

Adopting the ideas of Northrop, Kunitz & Herriot it is possible to offer the following very tentative explanation of the disappearance of the original inoculum. We may suppose that in the normal cell the nucleoprotein proteinogen is synthesized from nutrient materials by a catalytic reaction which is non specific in that it can potentially produce any nucleoprotein. Synthesis is probably effected by some relatively large aggregate of interdependent enzymes. The cell proteinogen will consist of two parts, one of which is combined with the synthesizing mechanism and is directing the synthesis to produce replicas of itself, while a second part is freely distributed through the cytoplasm and is being converted into the various native proteins of the cell. It is known that the ribonucleic acid of cell cytoplasm is partly concentrated in the relatively large mitochondria, and partly distributed in much smaller particles—the microsomes. As a result of the fusion of its surrounding membrane with the cell membrane the elementary body on entry into the cell disintegrates into soluble antigen and agglutinin particles. The soluble antigen is a replicating nucleoprotein and may be expected to compete with the cell proteinogen for the protein synthesizing mechanism. If the amount of virus proteinogen (soluble antigen) introduced is sufficiently large the cell proteinogen will be displaced from its directing role and the subsequent nucleoprotein synthesis will be directed by the virus. The disappearance of soluble antigen is therefore due to its entering into firm union with the nucleoprotein synthesizing mechanism. If the hypothesis of Burnet & Lind is accepted both agglutinin and soluble antigen are regarded as replicating units and the disappearance of both units would be accounted for. On the writer's theory the disappearance of the agglutinin cannot be accounted for in this way. However if it is supposed that agglutinin is derived from soluble antigen these two agents are probably normally in equilibrium in cell cytoplasm so that the removal of soluble antigen from the system by adsorption to the nucleoprotein synthesizing mechanism will result in the conversion of agglutinin into soluble antigen, a reversal of the normal reaction. This reversal would require energy and might therefore only occur under *in vivo* conditions.

Maintenance of strain type on passage

There is no difficulty in accounting for the persistence of similar biological characters such as serological type on serial passage, if each character is supposed to be carried by a special replicating unit. If however there is only one replicating unit which is potentially able to produce all the different types of agglutinin, etc., then it becomes necessary to explain how the strain maintains the same characters on passage. The characters of the original inoculum must in some way be carried over to reappear in the newly-formed virus. It may be that a small amount of the agglutinin of the original inoculum survives and acts as a pattern for the production of agglutinin later, or alternatively the enzymic activities of the original inoculum may cause the appearance in the cells of products of enzyme action which stimulate the production of the same enzyme later. The production of adaptive enzymes in bacteria may be stimulated by the presence either of the substrate or the products of the enzyme action.

Incomplete growth cycles

Most of the work on the virus growth cycle has been done with strains of virus highly adapted to growth in the host cell used, but very interesting results may be obtained in experiments in which the strain is not well adapted to the host cell. In such cases an incomplete growth cycle may be encountered in which the preliminary stages of multiplication may occur but the cycle is arrested before the appearance of fully infective virus. Schlesinger (1950) showed that when mouse brain was inoculated with a non-neurotropic strain of virus production of complement fixing antigen and haemagglutinin occurred but there was no production of infective virus. The yield of incomplete virus was proportional to the amount of infectious virus originally inoculated.

Incomplete growth cycles are sometimes encountered in eggs when the virus used is not egg-adapted. Thus when the D.S.P. strain of virus A which has been used in most of the writer's work was first isolated from man at the first egg passage no virus could be demonstrated by haemagglutination tests, but complement fixing antigen was readily detected. Similar phenomena have sometimes been seen when strains of virus B adapted to growth in the amniotic sac were transferred to the allantoic sac. On several occasions production of complement fixing antigen occurred in the chorio-allantoic membrane but the yield of haemagglutinin was negligible and the allantoic fluid failed to infect eggs on further passage.

Even with highly adapted virus incomplete cycles may occur if the inoculum used is large (von Magnus, 1951*b*). The usual result of such a procedure is the production of a large amount of non-infective agglutinin. This probably results from a premature disintegration of the cytoplasm of the host cell before the intracellular cycle is complete.

Problem of adaptation, variation and mutation

When influenza virus from man is introduced into a fertile egg growth may be obtained only with difficulty, but with serial passage the strain becomes adapted, and growth becomes more rapid and the yield of virus increases. Definite changes in the biological properties of the virus occur—the O-D variation. Burnet regards this process of adaptation as due to the selection of a mutant present in small amount in the original virus population, the mutant being better adapted for growth in the egg than the original strain. The variation only occurs if passages are made with large inocula, if inoculations are made at dilutions close to the limits of infectivity the characters of the original strain are maintained. This suggests that the variation is not due to gradual adaptation to a new environment but is due to the selection of a pre-existing mutant.

Burnet believes that whenever a large population of virus particles develops from a single small inoculum a few particles are formed which differ from the majority. If any new biological property appears this must indicate the appearance of a new genetic unit, and Burnet regards the new strain as a mutant. But it is possible that the changes involved in adaptation and variation do not involve the appearance of new replicating units but represent the development from the same replicating unit of a new type of enzyme. The new strain is not a mutant but a variant. It would be expected that the development of a new type of enzyme would be due to some effect of the environment, and in fact Magill & Sugg (1948) have suggested that the O-D change is to some extent dependent on the environmental conditions.

Recombination of strain characters, production of hybrid strains

Burnet & Lind (1951*a*, *b*) showed that when cells were infected simultaneously with two different strains of influenza virus, under suitable conditions it was possible to recover a new strain the properties of which were partly derived from one of the original strains and partly from the other. Similar phenomena occur with bacteriophage and these observations afford very strong evidence that in the course of intracellular growth virus becomes disintegrated into smaller units and is later reformed by a process of aggregation or recombination of the smaller units. In this way it is possible for the characters of one strain to become transferred to the other.

Recombination however occurs only between closely related viruses. With viruses which are not closely related interference occurs and one virus suppresses the other.

Burnet & Lind suggest that for recombination to occur the genetic units which become interchanged must have allelomorphic relations with each other. The writer's hypothesis offers perhaps a better explanation. It might be that recombination of characters is only possible between strains having a common replicating unit, while if the replicating units are different interference occurs. It may be possible to classify viruses in this way, viruses showing the phenomenon of recombination being regarded as variants of the same species.

The interference phenomenon

The interference phenomenon is one of the most remarkable characteristics of virus growth and must be accounted for by any satisfactory theory of virus multiplication. It is becoming increasingly evident that interference between viruses is a complex phenomenon. The initial union of virus and cell is due to the union of viral agglutinin with cell receptors. Destruction of the cell receptors will prevent infection of the cells (Stone, 1948). Inoculation of eggs with large doses of virus may result in total destruction or blocking of the cell receptors so that a second virus introduced later may fail to infect the cells. This phenomenon probably accounts for the interference between viruses inactivated by ultraviolet light or by heat and active virus introduced later (Zeigler, Lavin & Horsfall, 1944). The non-infective agglutinin which is found in the allantoic fluid of eggs inoculated with a large dose of virus (Gard & von Magnus, 1946) probably interferes with the growth of active virus in the same way. In all these cases a relatively large amount of the interfering agent is necessary to prevent multiplication of active virus. But it is not certain that destruction or blocking of cell receptors entirely accounts for the interfering powers of various types of non-infective or inactivated virus with the homologous active virus (Henle & Henle, 1947), and it seems most unlikely that it can account for the interference between totally distinct viruses such as influenza A and B (Ziegler & Horsfall, 1944). In these cases the cell receptors are not identical and total destruction of the cell receptors for one virus could not prevent infection by the other. It seems necessary to look for some intracellular effect and it seems probable that the phenomena are to be explained by the interference between replicating units discussed above. Active virus introduced into a cell appears to combine with the nucleoprotein synthesizing mechanism and a second virus introduced later may be unable to displace the first virus from its role in directing the course of nucleoprotein synthesis.

A third method of interference is also possible. Infectivity appears to be to some extent a function of the size of the infecting particle. Thus although infected allantoic fluid contains a large range of particles of virus protein from 120 A.U. diameter and upwards, it appears that particles of less than about 800 A.U. are non-infective. If active virus is growing in the cells and a large amount of a second virus is introduced later the enzymic effects of the second inoculum may produce so great a mechanical weakening of the cell wall that the cell begins to disintegrate prematurely and particles are released from the cell before much aggregation has occurred and before the normal intracellular cycle is complete. The effect would be similar to the phenomenon of 'lysis from without' shown by bacteriophage. The active virus would be released in the form of incomplete particles too small to show infectivity. The phenomenon of auto-interference, in which the yield of infective virus becomes reduced if the original inoculum is too large may be explained in this way.

Possible fusion of the theories of Hoyle and of Burnet & Lind

The two theories, although differing in some respects, have a great deal in common and it might not be too difficult to effect a compromise between them. One might suppose that each of the genetic units of Burnet & Lind existed in two states, a non-specific replicating state and an enzymically active but non-replicating state. On entry of the virus into the host cell the genetic units would be converted into the non-specific replicating state, in which they would be serologically indistinguishable but capable of replication. This state would correspond to the soluble antigen. Later the units would undergo a change of state, becoming enzymically active and serologically specific but losing the ability to replicate. Agglutinin would then appear in the cells.

However it is probably best to regard both theories as working hypotheses which suggest further lines of work rather than to attempt on inadequate data to decide between them or effect a compromise. This is especially so as the theories involve some rather different philosophical concepts.

Nature of the influenza virus

The writer regards the influenza virus elementary body as a detached fraction of the cytoplasm of the infected host cell. The detaching of portions of cytoplasm from cells may well be a very frequent or even a normal phenomenon in the course of the life of the cell, but not all such fractions will show virus activity. In order to show the activities of a virus the cell fragment must fulfil two conditions, it must be able to enter another cell and it must contain a sufficient amount of a replicating nucleoprotein to be able to reproduce itself on entry into that cell.

These conditions will be best fulfilled if the replicating nucleoprotein is itself able to produce an enzyme attacking the cell wall. The production of such an enzyme will enable the replicating unit to escape from the cell and will facilitate its entry into a second cell. It is becoming increasingly clear that very many viruses fulfil these two conditions. All viruses appear to consist largely of nucleoproteins, and the production of 'soluble antigens' in the infected cell is an outstanding characteristic of virus multiplication. The production of enzymes attacking cell walls, at first thought to be peculiar to the influenza group of viruses, is steadily being extended to other viruses and may well prove to be a general phenomenon. The toxic and disease producing activities of viruses may be largely a result of the actions of these enzymes.

SOME PHILOSOPHICAL CONSIDERATIONS

We have seen that Burnet regards the process of adaptation and variation as due to the selection of pre-existing mutants, and that the production of such mutants in small numbers is characteristic of virus multiplication. Such a process of mutation must represent the appearance from time to time of entirely new types of genetic unit, since it can scarcely be believed that every virus elementary body includes at least one example of all possible genetic units. In the general field of biological evolution also new types of genetic unit must appear from time to time, the only possible alternative being to believe that the original 'primeval cell' included one molecule of every possible replicating nucleoprotein.

There are only two ways in which a new type of genetic unit or a new type of replicating nucleoprotein can appear. It might be produced by modification of a previously existing unit as a result of some interaction with the environment, or it may be created *de novo*.

It may be impossible to decide between these two alternatives. Thus in order to demonstrate that a virus population includes a small number of mutant forms it is necessary to carry out some experiment designed to isolate the mutants, and it is impossible to be sure that the experiment does not itself cause the appearance of the mutant. This is of course the old difficulty that the act of observing a phenomenon is itself a part of the phenomenon, and that the observer is himself a part of the world he observes. However Burnet specifically denies that mutants are produced by an adaptation to a new environment, and it would seem that his concept involves the idea of the continuous creation of new types of genetic unit. Mutation is elevated to the status of a natural law. But if we are to believe that, whenever a large number of virus particles develops from a single original one, one particle is different from all the others, we are then faced with the tremendous problem of who or what decides which one.

The writer suggests that adaptation and variation is not due to the appearance of new types of replicating unit, but to the development from the same replicating unit of new types of enzyme or other active molecule as a response to environmental conditions. The concept of self-replication becomes meaningless if the new units produced are not in fact replicas of the original. Some form of constant invariable basic unit seems to be necessary to introduce order into the biological world, in the absence of such units the living world would become a chaos. In contrast with Burnet's view the writer's suggestion is to some extent in conflict with Darwinian theory. If new characters appearing in the process of adaptation are produced in response to changes of environment and are maintained on passage we have an example of the inheritance of an acquired character.

The idea, while admitting the possibility of great variation in individual viruses or in individual biological species, seems to deny the possibility of mutation. It might be possible to classify the various biological species in terms of types of replicating nucleoproteins present in their cells, but if these molecules are normally invariable it may become necessary to invoke creation to account for the existence of different species. But in this case creation is not a continuous process but an occasional or intermittent one.

Biological multiplication is a phenomenon which is only found in cells. Cells must contain self-replicating molecules, but these molecules do not themselves carry all the enzymes necessary for their own synthesis. We are faced with a system in which nucleoprotein synthesis is dependent on the presence of enzymes which can themselves only be produced from previously synthesized nucleoprotein precursors or genes. Such a system can only persist if the various interdependent units and the nutrient materials from which they are built are kept in close association by being enclosed in some form of semi-permeable membrane, and the membrane itself must be produced by preformed enzymes. All eternity might not be long enough for such an extraordinary system to arise by chance in an inanimate world, and we are forced to the conclusion that somewhere at some time a cell must have been created. It is difficult or impossible to visualize any simpler system than the cell which could show all the characters of living matter. Viruses and enzymes show some of the characters of life but are incapable of independent multiplication and cannot be regarded as intermediate stages in the evolution of living from non-living matter, but must be regarded as fractions of living cells. Between the living cell and inanimate matter there exists a gulf over which creation affords the most intellectually satisfying bridge.

REFERENCES

BAUER, D. J. (1949). Multiplication of the animal viruses. *Nature*, **164**, 767.

BURNET, F. M. (1948). The initiation of cellular infection by influenza and related viruses. *Lancet*, 1, 7.

BURNET, F. M. (1951*a*). A genetic approach to variation in influenza viruses. 1. The characters of three substrains of influenza virus A (WS). *J. Gen. Microbiol.*, **5**, 46.

BURNET, F. M. (1951*b*). A genetic approach to variation in influenza viruses. 2. Variation in the strain NWS in allantoic passage. *J. Gen. Microbiol.*, **5**, 54.

BURNET, F. M. & LIND, P. E. (1951*a*). A genetic approach to variation in influenza viruses. 3. Recombination of characters in influenza virus strains used in mixed infections. *J. Gen. Microbiol.*, **5**, 59.

BURNET, F. M. & LIND, P. E. (1951*b*). A genetic approach to variation in influenza viruses. 4. Recombination of characters between the influenza virus A strain NWS and strains of different serological subtypes. *J. Gen. Microbiol.*, **5**, 67.

DARLINGTON, C. D. (1944). Heredity, development and infection. *Nature*, **154**, 164.

EDDY, B. E. & WYCKOFF, R. W. G. (1950). Influenza virus in sectioned tissues. *Proc. Soc. Exp. Biol. Med.*, **75**, 290.

FAZEKAS DE ST. GROTH, S. (1948). Viropexis, the mechanism of influenza virus infection. *Nature*, **162**, 294.

FAZEKAS DE ST GROTH, S. & DONNELLY, M. (1950). Studies in experimental immunology of Influenza. II. Production of viral antigens. *Aust. J. Exp. Biol. Med. Sci.*, **28**, 31.

GARD, S. & VON MAGNUS, P. (1946). Studies on interference in experimental influenza. II. Purification and centrifugation experiments. *Ark. Kemi Min. Geol.*, **24 B**, No. 8.

GULICK, A. (1938). What are the genes? II. The physio-chemical picture; conclusions. *Quart. Rev. Biol.*, **13**, 140.

HENLE, W. (1949*a*). Studies on host—virus interactions in the chick embryo—influenza virus system. I. Adsorption and recovery of seed virus. *J. Exp. Med.*, **90**, 1.

HENLE, W. (1949*b*). Studies on host—virus interactions in the chick embryo—influenza virus system. II. The propagation of virus in conjunction with the host cells. *J. Exp. Med.*, **90**, 13.

HENLE, W. & HENLE, G. (1947). The effect of ultraviolet irradiation on various properties of influenza viruses. *J. Exp. Med.*, **85**, 347.

HENLE, W. & HENLE, G. (1949). Studies on host—virus interactions in the chick embryo—influenza virus system. III. Development of infectivity, haemagglutination, and complement fixation activities during the first infectious cycle. *J. Exp. Med.*, **90**, 23.

HIRST, G. K. (1942). The quantitative determination of influenza virus and antibodies by means of red cell agglutination. *J. Exp. Med.*, **75**, 47.

HIRST, G. K. (1948). The nature of the virus receptors of red cells. I. Evidence on the chemical nature of the virus receptors of red cells and of the existence of a closely analogous substance in normal serum. *J. Exp. Med.*, **87**, 301.

HOYLE, L. (1948). The growth cycle of influenza virus A. A study of the relations between virus, soluble antigen, and the host cell in fertile eggs inoculated with influenza virus. *Brit. J. Exp. Path.*, **29**, 390.

HOYLE, L. (1950). The multiplication of influenza viruses in the fertile egg. *J. Hyg.*, **48**, 277.

HOYLE, L. (1952). Structure of the influenza virus. The relation between biological activity and chemical structure of virus fractions. *J. Hyg.* (In the press.)

HOYLE, L., REED, R. & ASTBURY, W. T. (1952). Electron microscope studies of the structure of the influenza virus. (In the press.)

LANGMUIR, I. & SCHAEFER, V. J. (1938). Activities of urease and pepsin monolayers. *J. Amer. Chem. Soc.*, **60**, 1351.

LIU, O. C. & HENLE, W. (1951). Studies on host—virus interactions in the chick embryo—influenza virus system. IV. The role of inhibitors of haemagglutination in the evaluation of virus multiplication. *J. Exp. Med.*, **94**, 269.

LURIA, S. E. (1950). Bacteriophage: An essay on virus reproduction. *Science*, **111**, 507.

MAGILL, T. P. & SUGG, J. Y. (1948). The reversibility of the O-D type of influenza virus variation. *J. Exp. Med.*, **87**, 535.

VON MAGNUS, P. (1951*a*). Propagation of the PR8 strain of influenza A virus in chick embryos. I. The influence of various experimental conditions on virus multiplication. *Acta path. microbiol. Scand.*, **28**, 250.

VAN MAGNUS, P. (1951*b*). Propagation of the PR8 strain of influenza A virus in chick embryos. II. The formation of 'incomplete' virus following inoculation of large doses of seed virus. *Acta path. microbiol. Scand.*, **28**, 278.

VON MAGNUS, P. (1951*c*). Propagation of the PR8 strain of influenza A virus in chick embryos. III. Properties of the incomplete virus produced in serial passages of undiluted virus. *Acta path. microbiol. Scand.*, **29**, 157.

MURPHY, J. S., KARZON, D. T. & BANG, F. B. (1950). Studies of influenza A (PR8) infected tissue cultures by electron microscopy. *Proc. Soc. Exp. Biol. Med.*, **73**, 596.

NORTHROP, J. H., KUNITZ, M. & HERRIOT, R. M. (1948). *Crystalline enzymes.* Columbia University Press, p. 232.

PAULING, L. (1940). Theory of the structure and process of formation of antibodies. *J. Amer. Chem. Soc.*, **62**, 2643.

SCHLESINGER, R. W. (1950). Incomplete growth cycle of influenza virus in mouse brain. *Proc. Soc. Exp. Biol. Med.*, **74**, 541.

STANLEY, W. M. (1938). The reproduction of virus proteins. *Amer. Naturalist.*, **72**, 110.

STONE, J. D. (1948). Prevention of virus infection with enzyme of *V. cholerae. Aust. J. Exp. Biol. Med. Sci.*, **26**, 287.

TROLAND, L. T. (1917). Biological enzymes and the theory of enzymic action. *Amer. Naturalist*, **51**, 321.

WYCKOFF, R. W. G. (1951). Electron microscopy of chick embryo membrane infected with PR8 influenza. *Nature*, **168**, 651.

ZIEGLER, J. E. & HORSFALL, F. L. (1944). Interference between influenza viruses. I. The effect of active virus upon the multiplication of influenza viruses in the chick embryo. *J. Exp. Med.*, **79**, 361.

ZIEGLER, J. E., LAVIN, G. I. & HORSFALL, F. L. (1944). Interference between influenza viruses. II. The effect of virus tendered non-infective by ultraviolet radiation upon the multiplication of influenza viruses in the chick embryo. *J. Exp. Med.*, **79**, 379.

DISCUSSION

L. HOYLE in introducing his printed paper: I apologize for the use of the term 'soluble antigen'. The antigen was first demonstrated in 1936 and the term 'soluble' was used because the antigen could not be sedimented in the high speed centrifuges available at that time. The antigen has a particle size of 120 Å.

The disintegration of the infective elementary body by ether treatment produces particles of 120 Å of two types, one identical with soluble antigen and one with red cell agglutinating properties. The soluble antigen appears to be a ribonucleoprotein while the agglutinin is an enzyme.

In growth cycle experiments infective virus entering the cells of the chorio-allantoic membrane rapidly disappears and no infective virus can be found in the cells until six hours later when from 30 to 100 times the original amount is excreted into the allantoic fluid. It is therefore evident that there is a non-infective intracellular phase distinct from the infective elementary body.

There is an initial phase between 1 and 2 hours after inoculation in which no virus can be demonstrated in the cells by any method, followed by the appearance at 2 hours of soluble antigen in the cells. This soluble antigen rapidly increases but my statement that the increase is at first logarithmic has been questioned by Dr Gard who pointed out that a regular linear increase fitted better with the observations. I agree that

Dr Gard is right and withdraw the statement that the increase is logarithmic.

There is an appearance and increase in amount of non-infective haemagglutinin in the cells 1·5–2 hours after the appearance of soluble antigen. Liu and Henle have maintained that non-infective haemagglutinin really appears at the same time as soluble antigen but that an inhibitor of red cell agglutination present in the chorio-allantoic membrane prevents the detection of haemagglutinin in the early stages. Treatment of the membranes with R.D.E. removed this inhibitor effect. I am unable to accept this view. I have studied the matter:—

(*a*) by determining the amount of inhibitor in normal C.A.M. and the effect which this has in preventing recovery of haemagglutinin.

(*b*) by determining the length of time necessary to incubate the membranes *in vitro* in order to get the maximal release of haemagglutinin. A period of 6 hours permits the haemagglutinin present to destroy the inhibitor.

(*c*) by the use of R.D.E.

After the use of all three methods I still found that the soluble antigen appeared first. It is necessary to make some allowance for the very great sensitiveness of the haemagglutination test in relation to complement fixation and this is best done by measuring the amounts of the various agents in terms of the amounts present in the original virus inoculum.

Whereas in the chorio-allantoic membrane at 5 hours soluble antigen and non-infective haemagglutinin are present as separate units, in the allantoic fluid at 7 hours these agents are present in a single unit—the elementary body. This is to be explained by the release from the cell at 6 hours of small portions of cytoplasm enclosed in cell membrane. These become converted into elementary bodies by loss of water.

The advantage is obvious of accepting as the intracellular phase of the virus, something—the soluble antigen—which really is there at the relevant time, rather than inventing some hypothetical unit for which there is no evidence.

It is unnecessary to suppose the existence of a large number of different genes in the virus. The elementary body contains two units, the nucleoprotein soluble antigen which is always the same, and the enzymically active agglutinin which varies in different strains. With the aid of these two units it is possible to explain almost all that is known about influenza virus.

S. GARD: Experiments on Theiler's virus have shown that virus can be firmly fixed to the tissue debris in which case only a minor part of the total amount of virus can be eluted by extraction with ordinary saline. However, after treatment of the residue with proteolytic enzymes like ficin or with *M* NaCl large amounts of virus are liberated. Phenomena of this type might make the assay of intracellular virus in the course of multiplication technically difficult. The fact ought to be borne in mind in discussions concerning the time relationships in the development of different stages of a virus.

H. J. F. CAIRNS: Before accepting the idea that soluble antigen is a precursor of the influenza virus particle, it is necessary to consider what legitimate methods are available for comparing the times of appearance of different virus activities. For purposes of the present argument, the appearance of virus can be compared to the discharge of billiard balls from a sack. Suppose that, in this sack, there are 100 red balls for every white ball. When the sack discharges its contents, the first detectable change observed outside the sack will probably be the appearance of a red ball, since red balls are in a great majority; this finding, by itself, would be insufficient basis for the conclusion that red balls are discharged before white balls and thence that red balls become white balls some time after their discharge. If an observer wishes to state that the discharge of red balls tends to precede that of white balls, he must first show that the proportion of the red balls, which is discharged during the early stages, is greater than the proportion of the white balls, discharged in the same period; in this particular case, the number of red balls discharged in each period would have to be divided by 100 before direct comparison could be made between the number of balls of each colour discharged in each period. Translating this principle into terms of virus multiplication, the number of billiard balls of a particular colour, determining as it does the sensitivity of the observer's test for release of balls of that colour, is equivalent to the magnitude of the single cycle rise in titre of a particular component above its base-line. Where titres are plotted logarithmically, as is usually the case, it is sufficient to set all scales so that the final first-cycle yields of all components coincide; any level below this final yield (now common to all components) will represent the same proportionate yield for each component. When this procedure is applied to the results published by Hoyle, Henle, and others, it becomes quite obvious that the same proportions of the final yields of all components appear in each time interval. Here, it is sufficient to demonstrate the point using the figures, published by Hoyle (*J. Hyg.*, 1950, **48,** 277), for the appearance of soluble antigen and virus antigen in the allantoic membrane (see Fig. 1 on p. 246). Results dealing with the appearance of haemagglutinin and infective virus can be found elsewhere (Henle & Henle, *J. Exp. Med.*, 1949, **90,** 23).

Thus the completion of influenza virus multiplication is marked by the synchronous appearance, on the one hand, of soluble antigen, on the other, of infective haemagglutinin and, in certain instances, non-infective haemagglutinin (the incomplete 'precursor' of Gard and Magnus). There is no more evidence for believing that either soluble antigen or incomplete haemagglutinin is a normal precursor of the virus particle than there is for believing, for instance, that the virus particle, once completed, forms soluble antigen. Perhaps the most reasonable hypothesis to hold at the moment is that incomplete haemagglutinin and soluble antigen are, like the virus particle itself, *products* of virus multiplication, the former being the end-product of a particular type of defective virus multiplication, the latter being mainly an inevitable by-product whose subsequent function, if any, remains unknown.

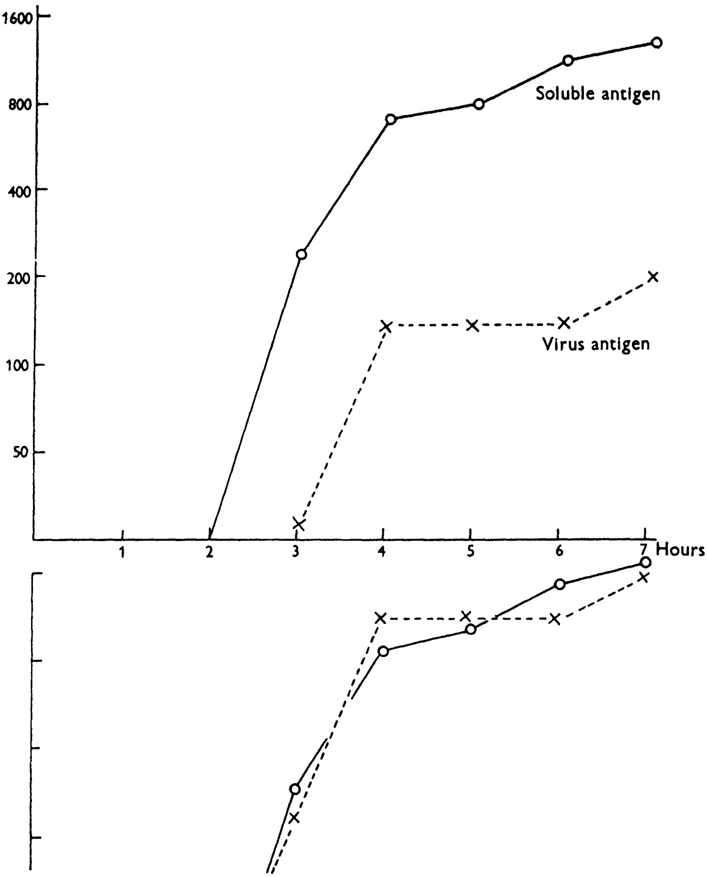

Fig. 1

Upper Graph: The appearance of soluble antigen and large particle (virus) antigen in the allantoic membrane (after Hoyle, 1950). These results were taken to indicate that the production of soluble antigen occurs about one hour in advance of the production of virus antigen. As plotted, the base-line represents about 2·5 % of the yield of soluble antigen and 17 % of the yield of virus antigen.

Lower Graph: The same results plotted so that the final yields of both components coincide. It can be seen that approximately 75 % of the first-cycle yield of both components appears between the 3rd and 4th hour.

J. B. BROOKSBY: I refer to work carried out with my colleagues at Pirbright on the application of more precise ultracentrifugation methods in the analysis of the virus system in foot-and-mouth disease. Detailed discussion of these methods and some of the results will be published shortly (Bradish, Brooksby, Dillon & Norambuena, *Proc. Roy. Soc.* B., 1952, in the press). It has been found possible to estimate the sedimentation constants of the components present in virus preparations by centrifugation in inclined tubes in angle rotors and the results have been correlated with those obtained in radial tubes. It follows that it is possible to determine theoretically the conditions for separation of two components on a preparative scale, when the sedimentation constants are known.

In the case of foot-and-mouth disease there is evidence for the existence of two particles, the first having a sedimentation constant of 70 *S* and being associated with the infectivity of the material and up to half of the total complement-fixing activity. The second particle has a sedimentation constant of 8 *S* and is associated with the remaining complement-fixing activity and certainly not more than 1 : 10,000 of the infectivity. A single centrifugation under appropriate conditions in either radial or inclined tubes will give an estimate of the proportions of the two components, or what has been termed the 'partition' of the material. This accurate analysis of the partition of a biological activity between the components should contribute much to the study of both growth and disintegration of the virus.

It has been found, for example, that storage at −20° or heating at 56° both lead to a breakdown of the larger component to the smaller. In Table 1 is shown the result of heating on the products of partition of a virus suspension.

Table 1. Influence of heating at 56° on complement-fixing components in the virus of foot-and-mouth disease

	Initial Activity (per cent of original)	Activity after heating for	
		15 and 30 minutes	1 and 2 hours
Starting material	100	—	—
Supernatant (smaller component)	50	34	30
Deposit (larger component) ...	55	71	76

After heating, the activity of all samples, shown in columns 3 and 4, was due to small component. It is apparent that heat treatment has decreased the activity of the small component by about 40% while the activity of large component on conversion to small has increased by about the same amount.

Work so far on growth and multiplication has been limited. Two growth curve experiments carried out in cattle have demonstrated the similarity of the curves obtained for the development of infectivity and total complement-fixing activity. When the latter was partitioned, it was found that during the first 12 hours after inoculation (that is, until the maximum infectivity is reached) the ratio of the amount of smaller component to larger did not vary significantly from 35%. It is remarkable that the constant proportion of smaller component to larger should persist over the range of 10,000-fold increase in infectivity which took place during growth.

L. HOYLE: The suggestion made by Dr Fulton that the soluble antigen is the surface antigen of the infective particle must be wrong. The soluble antigen has no affinity at all for red cells and it would be impossible for the virus to agglutinate red cells if it was covered with a layer of soluble antigen 120 Å thick. The agglutinin must be on the surface.

Dr Cairns' argument is based on incomplete data and false premises. The total final yield of virus components cannot be assessed from measurements of the amounts present in the chorio-allantoic membrane, since much of the products are excreted into the allantoic fluid. This material must be included, and as the relative proportions of soluble antigen, specific antigen, agglutinin and infective virus are very different in the allantoic fluid and in the membrane, the inclusion of the excreted material will completely alter the proportions found.

There is also no reason to suppose that the specific complement-fixing antigen is specially characteristic of a complete virus particle. The infective elementary body includes both specific and soluble antigen, and both these antigens may be found in non-infective particles of smaller size. The specific antigen is closely related to and probably identical with the haemagglutinin.

The growth of virus in cells is in no way comparable to the discharge of billiard balls from a sack. It is a quite unjustified assumption that the ratio of the various components present at the end of the cycle is the same as that in the original inoculum. The ratio in the elementary body is the same, but the elementary body is not the only product of virus multiplication. The main product is soluble antigen, much of which is never incorporated into elementary bodies at all.

The ratio of soluble antigen to specific antigen in the original elementary bodies entering the cells is about 5 : 4. If Dr Cairns' argument is correct this ratio should be maintained throughout the cycle. But at three hours in the membrane the ratio is 8 : 1 and at 6 hours it is 6 : 1. This indicates that the soluble antigen is produced first.

We are dealing with observed facts and not with statistical theories, with living virus protein and not with balls. Dr Cairns' statement that the completion of virus multiplication is marked by the synchronous appearance of soluble antigen and infective haemagglutinin is untrue. No statistical analysis will alter the fact that an extract of the chorio-allantoic membrane made 3–4 hours after inoculation contains an amount of soluble antigen much greater than in the original inoculum, while it contains an amount of infective haemagglutinin less than 1% of that originally introduced.

Dr Brooksby's demonstration of the presence of complement-fixing antigens of different particle size in foot-and-mouth disease, and that the smaller component can be derived by disintegration of the larger infective unit affords an interesting parallel with influenza.

His growth curve experiment fails to demonstrate any time difference in the appearance of these units, but such a difference can only be demonstrated in single cycle experiments, and an experiment in which the infectivity rose 10,000-fold is almost certainly a multiple cycle experiment.

GROWTH OF VIRUSES OF THE INFLUENZA GROUP AS REVEALED BY ELECTRON MICROSCOPY OF INFECTED CELLS

T. H. FLEWETT

The School of Medicine, Leeds

The advent of new techniques by which tissue cells could be examined by the electron microscope has opened new lines of investigation of virus multiplication. Whereas until recently influenza and related viruses could be examined only after they had been purified by differential centrifugation, with the selection of the small range of particle sizes which differential centrifugation involves, they can now become adsorbed onto the membranes of haemolysed erythrocytes and photographed (Dawson & Elford, 1949). Likewise the components of tissue cells—mitochondria, microsomes, neurofibrils, cell membranes—can now be studied in their proper place within the cell, either when it has been cut into thin sections (Newman, Borysko & Swerdlow, 1949; Hillier & Gettner, 1950) or when it has spread its cytoplasm thinly in tissue culture on a plastic membrane (Porter, Claude & Fullam, 1945). It seemed feasible, using these various techniques, to try to get some direct evidence, in the form of photomicrographs, of how influenza and fowl plague virus behave during their growth, and if possible to obtain direct evidence either to confirm or deny the ingenious hypothesis advanced by Hoyle (1948, 1950) that influenza virus elementary bodies break up within the cell into large numbers of smaller self-replicating units which subsequently recombine to form fresh elementary bodies. This article describes work carried out by the author with Dr C. E. Challice at the National Institute for Medical Research during the last three years.

For these investigations the virus of classical fowl plague has chiefly been used. Its advantages are: (1) it is extremely pathogenic for the chick embryo; (2) it produces intranuclear inclusions which can easily be seen in living cells with the phase contrast microscope; (3) it produces in the egg elementary bodies and filaments of various lengths very similar in size and morphology to those associated with recently isolated strains of A-prime influenza virus (Dawson & Elford, 1949). The Melbourne Egg, PR8, A/Nederland 1/49 and A/Madrid 3/51 strains of influenza virus A were used for comparison.

TISSUE CULTURE EXPERIMENTS

Cultures of amniotic epithelium were made on plastic membranes mounted upon coverslips, which were inverted over hollow-ground slides and incubated as 'lying-drop' cultures. The nutrient medium used was normal amniotic fluid of 10–12 day-old chick embryos. This had the advantages of being optically clear and of having a very low protein content, so that when the preparations were eventually fixed with osmic acid solution, there was very little precipitation of protein upon the cells. The details of the method of preparing these cultures have been described by Flewett & Challice (1951*a*); their method was a modification of that extensively used by Wirth & Barski (1947) and Barski, Maurin & Croissant (1949). The cultures were incubated for two days to allow migration of cells around the explant. This migration was nearly always epithelial, fibroblasts in any number being rare. Some cultures of pieces of chick embryo lung were also made, and served as sources of fibroblasts. The preparations were then opened and inoculated with amniotic fluid from an egg inoculated amniotically 24 hours previously with the virus in question. In order to remove most of the filaments present in the fluid (Chu, Dawson & Elford, 1949) it was passed through a collodion filter of average pore diameter 700 $m\mu$. The filtrate was found to contain elementary spherical bodies, cocco-bacillary particles, and short filaments up to about 0·5μ in length. The cultures were then inoculated with this filtrate, and their development followed by phase-contrast microscopy. They were fixed by a solution of osmic acid after various intervals and mounted for examination by electron microscopy.

For comparison, roller tube cultures were made in tubes of 30 ml. capacity and about 2·5 cm. diameter. Each tube contained about one-third of a minced chorio-allantoic membrane and 4 ml. of White's synthetic medium (White, 1946). The tubes were rotated at 12 revolutions per hour within a closed box. 'Gassing' was done by passing various gas mixtures into the box, the constricted necks of the tubes being plugged with cotton-wool. The inoculum consisted of one drop of the filtrate used to inoculate the hanging drop cultures.

The appearance of living cells by phase-contrast microscopy closely resembles the appearance of the same cells when fixed with osmic acid solution and examined in the electron microscope. Contrast observed in phase-microscopy is due to optical path differences (usually refractive index) in the object, and in electron microscopy, contrast is due to differences in electron scattering (mass of atoms multiplied by number of atoms per unit area of specimen). Electron optical 'staining' by the heavy osmic acid used as fixative, of fat and other refractile cellular material makes the electron micrograph usually resemble the phase-contrast image in general intensity distribution.

The earliest change visible by phase-contrast microscopy after inoculation of cultures with fowl plague virus was an increase in the size of nucleoli in some of the cells after about 24 hours (Fig. 2; compare with the normal, Fig. 1). They become refractile and granular and further increased in size on continued incubation, until after 48 hours nearly all the nucleoli showed this change. Later still, numerous refractile granules appeared in the nucleoplasm (Fig. 3). At this stage, many small vacuoles, 2–3 μ in diameter, appeared in the cytoplasm, most of them around the nucleus. The enlarged nucleoli finally broke up more or less completely into granular fragments.

Up to the stage of the appearance of the refractile granules in the nucleoplasm, the mitochondria showed little change. In the first 24 hours after inoculation, they tended to become longer and thicker, and then smaller and shorter. When the vacuoles appeared they became less refractile, and finally, as the intranuclear inclusions broke up, they disappeared.

Forty-eight hours after inoculation some cells had disappeared from the outgrowth, and after 72 hours they had nearly all disappeared.

Similar cultures were inoculated with A/Nederland 1/49 influenza virus. No intranuclear inclusions were formed, but granular refractile structures appeared in the cytoplasm of some of the cells. These stained red with haematoxylin-eosin preparations of fixed cultures. Similar structures were however often found in control preparations.

No fowl plague virus was found in cultures examined by electron microscopy until 36 hours after inoculation, by which time intranuclear inclusions were already present. It was mainly in a filamentous form, the filaments frequently being in bundles; a few of them showed segmentation into elementary bodies. Fig. 4 shows filaments apparently either emerging from the nucleus or overlying it. It was difficult to be sure whether the filaments were within the cell or upon its surface, since when the cell was thin enough for virus to be resolved, it was thinner than the depth of the focus of the microscope. A few preparations were shadowed; but as Porter, Claude & Fullam (1945) showed, the cytoplasm of a thinly spread cell, when finally mounted, may be thinner than a virus filament, so that the presence of a shadow need not mean that the virus was upon the cell surface rather than within it. However, the way in which the contents of the vacuole (Fig. 4) have contracted away from the virus on drying strongly suggests that the virus here was actually intracellular.

Other preparations of both fibroblasts and epithelium 48 hours after inoculation showed filaments apparently undergoing segmentation into elementary bodies (Fig. 5). Similar pictures of influenza virus both in tissue cultures and sections have been published by Murphy, Karzon & Bang (1950), Eddy & Wyckoff (1950), and Wyckoff (1951), who also believed that the filaments underwent segmentation.

EXAMINATION OF THIN SECTIONS: STRUCTURE OF FOWL PLAGUE INCLUSIONS

No matter how well-spread a tissue culture cell may be, its nucleus remains much too thick for the electron beam to penetrate it well enough to give a satisfactory image. In order to investigate further the nature of the fowl plague intranuclear inclusions, a technique of preparation of thin sections was developed (Flewett & Challice, 1951). The membranes of infected eggs were used, usually the amnion, which could easily be infected on both its amniotic and allantoic surfaces, by inoculation into the appropriate cavity.

The inoculum used was 0·05 ml. of pooled allantoic fluid harvested from eggs 18 hours after inoculation with 1/1000 dilution of allantoic fluid from another fowl-plague infected embryo (24 hours after inoculation in the case of influenza). This dose usually contained 10^8–10^9 infective particles. Assuming the average free fluid-washed surface of an allantoic or amnion lining cell to be 25 μ square, and that the infective particles distributed themselves randomly over the exposed surface, then 10^8 infective particles should suffice to infect the majority of the cells in the membrane.

0·2 μ sections were cut for electron microscopy, and at 0·6 μ for ultraviolet microscopy. Sections of membranes fixed in Carnoy's solution were stained by a pyronin-methyl green mixture (Bate, 1950).

Intranuclear inclusions appeared simultaneously in almost 100% of the cells lining the infected cavities about 10 hours after inoculation. They could easily be seen by phase-contrast microscopy in cleared unstained sections and could be demonstrated by haematoxylin and eosin or by Lendrum's stain (Lendrum, 1947) if differentiation was not carried too far. They presented the general features of Cowdry's type 'A' intranuclear inclusions. When stained by a methyl-green-pyronin mixture the inclusion consisted of a red centre, surrounded by a thin rim of green-staining material, while the bulk of the chromatin (staining green) lined the nuclear membrane. Direct and phase-contrast ultraviolet pictures ($\lambda = 276{\cdot}5$ mμ) taken of 0·6μ sections of material fixed in osmic tetroxide solution showed inclusions consisting of a centre with low absorbing power surrounded by a granular rim of highly absorbing material, essentially similar to the picture given by the methyl-green-pyronin stain (Figs. 6 and 7).

Sections were cut at 0·2μ and, after the wax had been caused to sublime away by gentle heating *in vacuo*, these were examined without shadowcasting in the electron microscope. Most of the preparations made were of tissue 17 and 22 hours after infection. The inclusions always showed a granular structure, the particles being of the same order of size as the elementary bodies, usually 50–200mμ (Fig. 8). In some nuclei, short filaments of about 100 mμ diameter were seen, and Fig. 8, shows a micrograph of a section which apparently went through the centre of an inclusion, suggesting that it consisted of one, or possibly two nucleoli close together, closely invested by a mass of particles between 50–150 mμ in size, some being oval in cross section, with dimensions about 80 × 120mμ. These structures would appear as a single inclusion body with the light microscope. No granular inclusions were seen in tissue less than 8 hours after inoculation, though the nucleoli sometimes appeared rather larger than normal.

CYTOPLASMIC CHANGES IN INFECTED CELLS

The first appearance of virus in fowl-plague infected cells was 5 hours after inoculation, when a few filaments were seen projecting from the surface. After 8 hours (Fig. 9) in sections of membranes infected either with fowl plague or A/Madrid 3/51 viruses, numerous filaments were seen projecting from the free surface of most of the cells and at about right angles to it. The filaments, as seen in section were usually up to about 0·5μ long; a few spherical structures were seen but may have been filaments cut transversely. Generally it was impossible to trace the filaments beneath the surface of the cell; the cell membrane was usually ill-defined and often could not be distinguished. A similar appearance was presented later; after 17 hours filaments were very numerous in sections of fowl-plague material. Fig. 10 shows the appearance 22 hours after infection with fowl plague; it is the only picture we have in which filaments were distinctly visible beneath the cell surface. At this stage the embryo was usually moribund, and most of the cells lining the allantoic and amniotic cavities had sloughed off. Elementary bodies were scanty in these sections, possibly because they were liberated more readily into the allantoic and amniotic fluids than were the filaments. Fig. 11, however, shows the surface of cells infected 48 hours previously with the Melbourne Egg strain of influenza virus A. This strain when examined after adsorption from allantoic fluid upon laked chick erythrocytes had few and short filaments compared with recently isolated A-prime influenza strains. Upon and just beneath the cell surface large numbers of elementary bodies and a few short filaments were seen, but no filaments could be made out in the depths of the cytoplasm. The cell membrane could not be made out, but in the cytoplasm there were filamentous mitochondria. The nuclei and cytoplasm otherwise showed no detectable difference from those of normal cells. Fig. 12 shows the appearance 64 hours after inoculation. The number of filaments relative to elementary bodies was greater; the nucleoli sometimes appeared larger than normal and some of the cells showed the presence of a cell membrane.

The fringe of virus filaments on the edge of infected cells could just be detected both in the direct and phase-contrast U.V. photomicrographs, although it could not be seen by visual phase-microscopy of the same sections.

Bate (1950) has described the occurrence of pyronin-staining granules in influenza-infected cells fixed in Carnoy's solution or alcohol. These granules appeared about 15 hours after infection, and grew in size and coalesced as the infection proceeded. The degree of granulation could be correlated with the amount of virus growth. It was thought that they represented an accumulation of ribosenucleic acid in the cytoplasm of infected cells. These results have been confirmed; it was found also that fowl-plague infected cells showed a very marked granulation when stained in this way. However, the granules could not be seen in any of the electron micrographs or in the osmic-fixed sections by visual or ultraviolet phase microscopy. Ribose nucleic acid strongly absorbs ultraviolet light, and produces strong phase retardation of visible light. It seems likely, therefore, that the granular appearance is an artefact due to coarse precipitation of ribose nucleic acid by certain fixatives.

It was impossible, from the micrographs described above, to be sure that they represented virus being liberated from infected cells, and not virus already liberated into the allantoic cavity entering fresh cells. In order as nearly as possible to reproduce the conditions obtaining in the cavities of an embryo 8 hours after inoculation by the method described above, eggs were inoculated as before with 0·05 ml. undiluted allantoic fluid and incubated for 8 hours. The allantoic and amniotic fluids were harvested separately. The amniotic and allantoic fluids from normal eggs were removed as completely as possible, and the amniotic and allantoic fluids from the eggs which had been infected for 8 hours were substituted. These eggs were then incubated for 1½ hours, when their membranes were fixed, embedded, and cut in sections 0·2μ thick. The results are shown in Fig .13. The cytoplasm appeared to have flowed ompletely or partly over the virus particles, and no cell membrane ould be clearly distinguished. Only a few such preparations have been nade, so one cannot say that this is a constant feature, or that virus ntering a cell can be distinguished from virus leaving a cell. It does, iowever, suggest that filaments may enter a cell, at any rate under onditions of an enormous infective dose as was given here. Fazekas de it. Groth (1948) suggested that this type of result might have been xpected, and gave the name 'Viropexis' to describe his hypothesis of iow influenza virus entered the allantoic cell.

GENERAL CONSIDERATIONS

t has always been found wise, when making observations with the light nicroscope to check the results of one staining procedure against those f another, and against the appearance of living cells. No such check an be made of electron micrographs of objects below 250 mμ in size, nd interpretation of micrographs of such complicated structures as thin ections of virus infected cells cannot be made with any certainty.

There are two discrepancies between the results of tissue culture experinents and the investigations on infected membranes in the egg. (1) The ime for new virus to appear, as seen by electron miscroscopy, was bout 36–48 hours in tissue cultures, and in eggs 5 hours. In tissue ultures intranuclear changes first became visible 24 hours after inculation, whereas in eggs only 10 hours incubation was needed. This iscrepancy does not appear, at any rate to the same extent, in roller ube cultures of fragments of either chorio-allantoic membrane or mnion inoculated with similar doses of fowl-plague virus. Here, the mount of supernatant fluid and especially the volume of air were very nuch greater than in the case of hanging drop cultures on a coverslip, nd the rate of growth of virus (measured as haemagglutinin) was not nuch slower and the length of time taken to produce extensive necrosis f the infected cells was not much longer (24–36 hours) than in the egg. 2) Virus as seen in tissue cultures with the electron microscope was lmost entirely filamentous, few elementary bodies being seen, and then nly in association with filaments, whereas in the allantoic fluids of eggs nd the supernatant fluids of roller-tube cultures both elementary bodies nd filaments of varying length could be discovered by the method of)awson & Elford (1949).

Similarly, in the few hanging drop cultures successfully infected with /Nederland 1/49 influenza virus, filament formation was extensive and ew elementary bodies were seen (Flewett & Challice, 1951*a*). Murphy, Karzon & Bang (1950) found many long filaments in hanging drop cultures infected with the PR8 strain of influenza A virus, which produces few and short filaments when growing in the egg, although I have been unable to repeat their results with this strain in tissue cultures.

The first appearance of fowl plague virus in sections was 5 hours after inoculation, and was in the form of filaments. At this time the first increase of haemagglutinin could be found in the allantoic fluid. Segmentation of filaments into elementary bodies was not seen in these sections, although Eddy & Wyckoff (1950) described segmented filaments in sections of mouse lungs infected with PR8 virus. Elementary bodies were rarely seen except in tissue infected with the Melbourne Egg strain of influenza A. One cannot however distinguish between spherical elementary bodies and transversely cut filaments as seen in sections.

The surface of the normal cells lining the allantoic and amniotic cavities of normal eggs is fairly smooth. Here and there can be seen small elevations of cytoplasm, conical in vertical section, rising to a height of up to 1 μ (Fig. 14). They are not easily confused with virus filaments, which are somewhat denser to the electron beam, and which have a constant diameter. The suggestion has been made by Wyckoff (1951) that influenza virus filaments might be built up on a scaffolding of normal cell components. Hoyle (1950), on the basis of observations made with the dark-ground microscope, has put forward similar suggestions. His observations were on normal and infected cells scraped off the lining of the chorio-allantoic membrane and suspended in saline, a procedure likely to damage them and to allow the outflowing of cytoplasm which might be seen as long thin threads. The studies here described do not support either hypothesis. A complex system of what looked like canals, about 100–150 mμ wide—possibly Golgi apparatus—was seen in the depths of the cytoplasm in normal cells lining the amniotic cavity, but it did not come to the surface where distinctive filaments are seen only in infected cells. *All* filaments are not virus, as Angulo (1951) pointed out, but it seems hard to doubt now that some filaments may be, and that the particles seen on the surface of infected cells are structures in their own right.

Our investigations have rarely demonstrated virus within the cytoplasm, whereas the brick-shaped elementary bodies of *herpes simplex* (Bang, 1950), fowl-pox (Lépine, Athanasiu & Croissant, 1948; Bang & Gey, 1950; Morgan & Wyckoff, 1950; *vaccinia* (Bang, 1950), and *molluscum contagiosum* (Banfield, Bunting, Strauss & Melnick, 1951) all have been demonstrated in the depths of the cytoplasm, where they appear to multiply. Distemper virus induces inclusions both in nuclei and cyto-

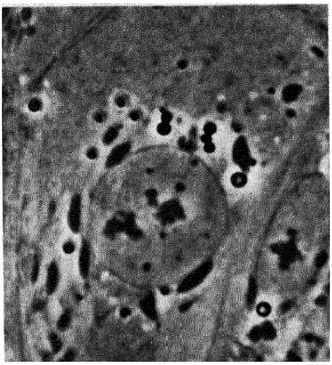

Fig. 1. A normal epithelial cell. Phase contrast, × 1,500. Photograph by Mr J. Smiles.

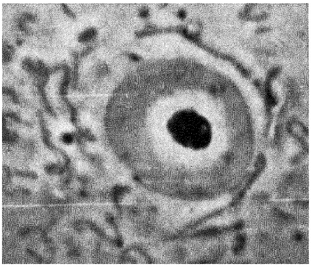

Fig. 2. Epithelium, 36 hr. after inoculation with fowl plague. Phase contrast, × 1,500.

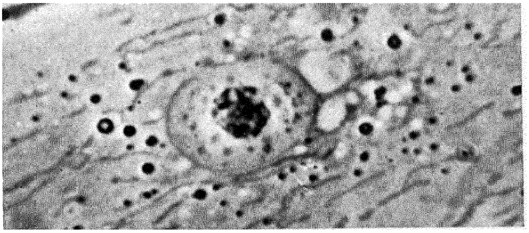

Fig. 3. Epithelium, 48 hr. after inoculation with fowl plague. Phase contrast, × 1,500.

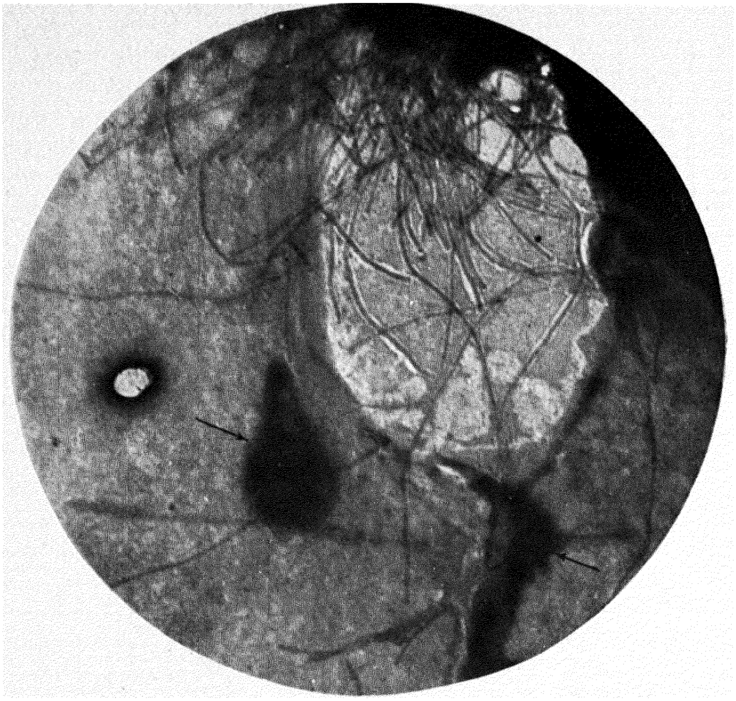

Fig. 4. Epithelium, 48 hr. after inoculation with fowl plague; filaments overlying or emerging from a nucleus. The arrow indicates a mitochondrion. × 10,000.

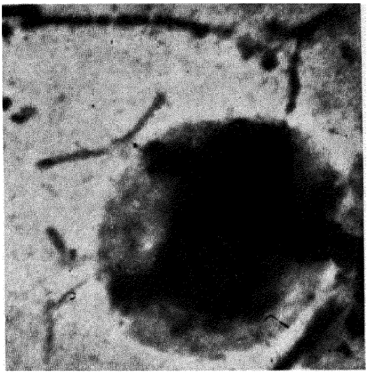

Fig. 5. Fibroblast, 48 hr. after inoculation with fowl plague; filaments show segmentation. The significance of the large round structure is unknown. × 22,000.

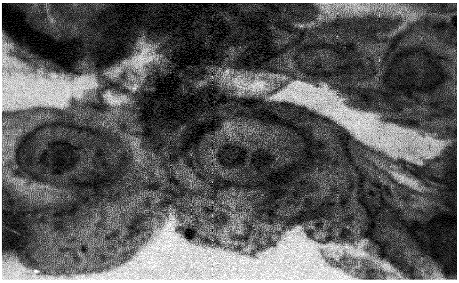

Fig. 6. Unstained section of amnion, 17 hr. after inoculation with fowl plague virus. Direct U.V. photomicrograph. × 2,500.

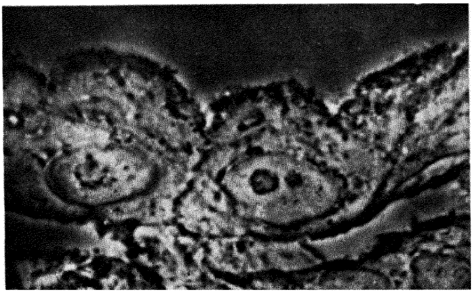

Fig. 7. The same; phase contrast ultraviolet photomicrograph. Figs. 6 and 7 by Mr J. Smiles. × 2,500.

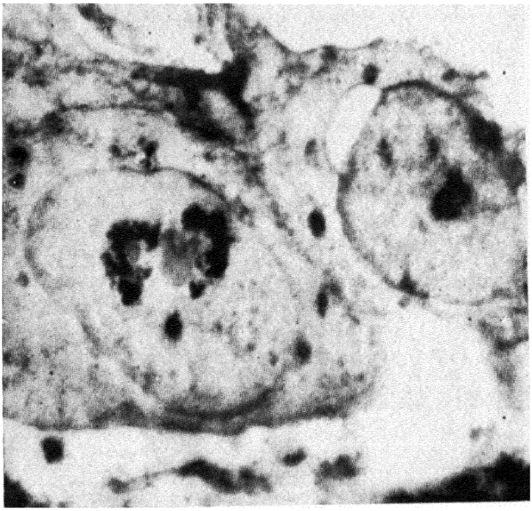

Fig. 8. 0·2 μ section through intranuclear inclusion; amnion, 17 hr. after infection with fowl plague. ×9,000.

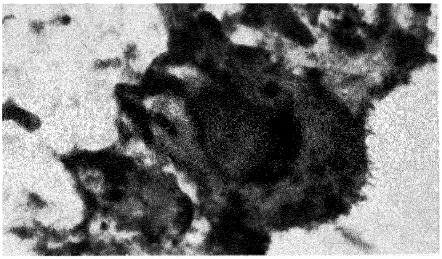

Fig. 9. Amnion, 0·2 μ section, 8 hr. after inoculation with influenza A/Madrid 3/51. × 7,000.

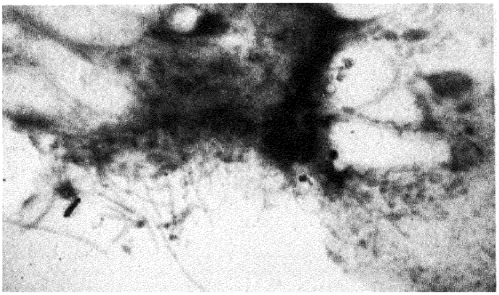

Fig. 10. Amnion, 0·2 μ section, 17 hr. after inoculation with fowl plague. × 6,000. Some filaments apparently in cytoplasm.

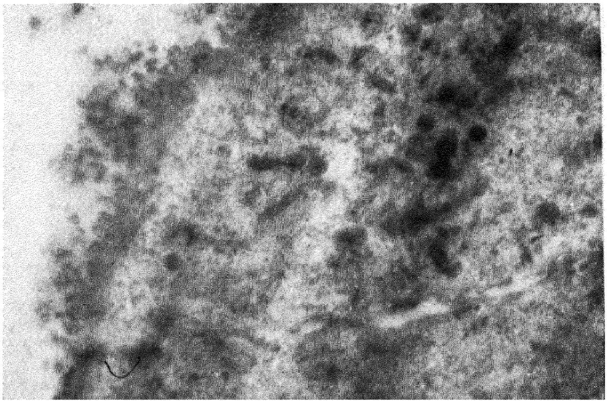

Fig. 11. Surface of allantoic cell, 48 hr. after inoculation with influenza A (Melbourne Egg). × 10,000.

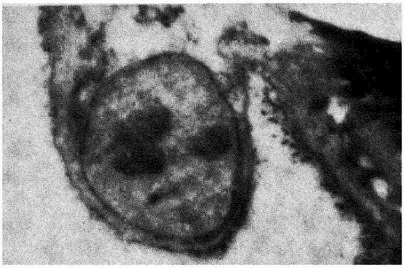

Fig. 12. Amnion, 64 hr., Melbourne Egg virus. A cell membrane appears to be present on part of the surface, × 7,000.

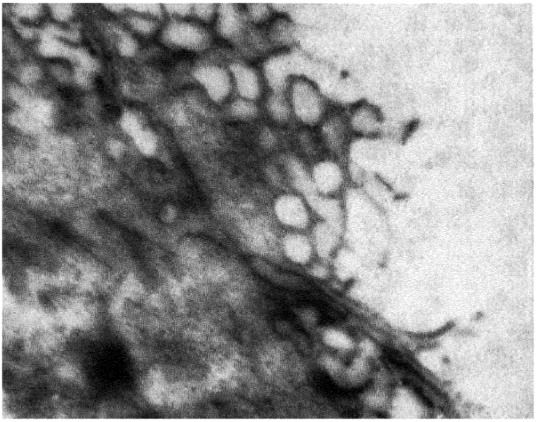

Fig. 13. Amnion, × 14,000. For description, see p. 254.

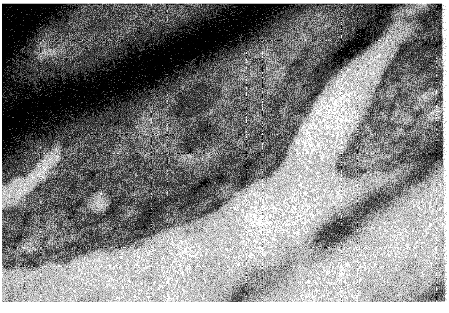

Fig. 14. Normal amnion. This cell more closely resembles one infected with influenza than any other examined. × 7,000.

plasm of infected cells, and so does yellow fever. It would not be possible to deduce from a few pictures that no virus is present in the form of particles or filaments within the cytoplasm of the cell. A filamentous structure is, of course, more distinctive than a spherical particle, but in the same way that it is unlikely that a filament will be sectioned along its length when it is protruding from the cell membrane, so the likelihood of sectioning along a filament orientated completely randomly within the cytoplasm is considerably less. Furthermore, the diameter of a structure such as a particle within a medium such as the cytoplasm as observed by electron microscopy is considerably less than when the particle is isolated (compare Fig. 4 in which filaments cross from a vacuole into cytoplasm). Nevertheless, from an examination of many sections of such material, both normal and infected, we are led to suggest that the influenza or fowl plague virus is not present in any great amount in the cytoplasm, at least not in the same form as when it is isolated from the host cell.

If one accepts this, i.e. that the virus is present only at the cell membrane, then one is led to seek some explanation. It seems possible that there may be some factor present in or about the cell membrane which is necessary for the growth of new influenza elementary bodies and filaments. This theory was proposed by Hoyle (1950). If so, it would allow the filaments observed in tissue cultures to be actually within the cell, for no object present in such a thin preparation could be far from the cell membrane.

Another discrepancy remains to be accounted for. Fowl plague virus began to be liberated into the allantoic cavity 5 hours after inoculation with the large dose of virus used in the experiments described, but intranuclear inclusions appeared in all the cells lining the allantoic cavity 10 hours after inoculation, where the virus haemagglutinin titre was already high in the allantoic fluid. Either the cells infected with the inoculum have liberated some virus and then, some 4–5 hours later, developed inclusion bodies, which implies that infected cells may liberate fowl plague virus without being immediately destroyed, or else the cells infected with the inoculum fail to develop inclusions and only those cells infected with freshly liberated virus develop inclusions. It may be that fowl plague virus kills the cells it infects by producing gross alterations within the nucleus, but how and why influenza virus kills the cells it infects remains open to conjecture. The use of the electron microscope has confirmed some theories previously suggested by work done by serological and other methods ; it may help to indicate further investigation along these lines.

Acknowledgements

I should like to thank Dr C. E. Challice for his collaboration in the work described here, Drs C. H. Andrewes, W. J. Elford and A. Isaacs, for their help, advice, and criticism, both of the work and the manuscript; Mr J. Smiles for the Figs. 1, 6 and 7, the Editors of the *Journal for General Microbiology* for permission to reproduce results and Figs. 1, 2, 4, 5 and the Editors of the *Discussion of the Faraday Society* for their permission to reproduce Figs. 8, 9, 10, 12, 13.

REFERENCES

ANGULO, J. J. (1951). On the identity of the so-called filamentous forms of influenza and fowl pest viruses. *Arch. ges. Virusforsch.*, **4,** 199.

BANFIELD, W. G., BUNTING, H., STRAUSS, M. J. & MELNICK, J. L. (1951). Electron micrographs of thin sections of *Molluscum contagiosum. Proc. Soc. Exp. Biol., N.Y.*, **77,** 843.

BANG, F. B. (1950). Cellular changes in the chick chorioallantoic membrane infected with herpes simplex and vaccinia. A study with thin sections for the electron microscope. *Johns Hopkins Hosp. Bull.*, **87,** 511.

BANG, F. B., & GEY, G. O. (1950). Experimental study of the virus-host cell relationship in fowl pox. *Fed. Proc.* **9,** 324.

BARSKI, G., MAURIN, J. & CROISSANT, O. (1949). Méthode de montage d' éléments cellulaires en vue de l'examen au microscope électronique. *Ann. Inst. Pasteur*, **76,** 1.

BATE, W. E. quoted by BURNET, F. M. (1950). *Ann. Rep. Walter and Eliza Hall Inst.*, Melbourne.

CHALLICE, C. E. (1951). Contribution to General Discussion. *Farad. Soc. Disc.*, **11.**

CHU, C. M., DAWSON, I. M. & ELFORD, W. J. (1949). Filamentous forms associated with newly isolated influenza virus. *Lancet*, **1,** 602.

DAWSON, I. M. & ELFORD, W. J. (1949). The investigation of influenza and related viruses in the electron microscope, by a new technique. *J. Gen. Microbiol.*, **3,** 298.

EDDY, B. E. & WYCKOFF, R. W. G. (1950). Influenza virus in sectioned tissues. *Proc. Soc. Exp. Biol., N.Y.*, **75,** 290.

FAZEKAS DE ST. GROTH, S. (1948). Viropexis, the mechanism of virus infection. *Nature*, **162,** 294.

FLEWETT, T. H. & CHALLICE, C. E. (1951*a*). The intracellular growth of fowl-plague virus. *J. Gen. Microbiol.*, **5,** 279.

FLEWETT, T. H. & CHALLICE, C. E. (1951*b*). The cutting of sections for electron microscopy, using ester wax as an embedding medium. *J. Path. Bact.*, **63,** 748.

HILLIER, J. & GETTNER, M. E. (1950). Improved ultra-thin sectioning of tissue for electron microscopy. *J. App. Phys.*, **21,** 889.

HOYLE, L. (1948). The growth cycle of influenza virus A. A study of the relations between virus, soluble antigen and host cell in fertile eggs inoculated with influenza virus. *Brit. J. Exp. Path.*, **29,** 390.

HOYLE, L. (1950). The multiplication of influenza viruses in the fertile egg. *J. Hyg.*, **48,** 277.

LENDRUM, A. C. (1947). The phloxin-tartrazine method as a general histological stain and for the demonstration of inclusion bodies. *J. Path. Bact.*, **59,** 399.

LÉPINE, P., ATHANASIU, P. & CROISSANT, O. (1948). Aspect au microscope électronique des inclusions de la variole aviaire (corps du Bollinger). *Ann. Inst. Pasteur*, **75,** 279.

MORGAN, C. & WYCKOFF, R. W. G. (1950). The electron microscopy of fowl pox virus within the chorio-allantoic membrane. *J. Immunol.*, **65**, 285.

MURPHY, J. S., KARZON, D. J. & BANG, F. B. (1950). Studies of influenza A (PRS) infected tissue cultures by electron microscopy. *Proc. Soc. Exp. Biol., N.Y.*, **73**, 596.

NEWMAN, S. B., BORYSKO, E. & SWERDLOW, M. (1949). New sectioning techniques for light and electron microscopy. *Science*, **110**, 66.

PORTER, K. R., CLAUDE, A. & FULLAM, E. F. (1945). A study of tissue culture cells by electron microscopy. *J. Exp. Med.*, **81**, 233.

RAKE, G. & BLANK, H. (1950). The relationship of host and virus in *Molluscum contagiosum*. *J. Invest. Dermatol.*, **15**, 81.

WHITE, P. R. (1946). Cultivation of animal tissues *in vitro* in nutrients of precisely known composition. *Growth*, **10**, 231.

WIRTH, J. & BARSKI, G. (1947). Cultures épithéliales sur membranes plastiques sans repiquages et sans plasma; préparation rapide de cultures colorables comme des frottis. *Ann. Inst. Pasteur*, **73**, 987.

WYCKOFF, R. W. G. (1951). Electron microscopy of chick embryo membrane infected with PRS influenza. *Nature*, **168**, 651.

DISCUSSION

N. W. PIRIE: The suggestion that you make that much of the virus is located in the cell wall interested me greatly. It was in fact the idea that virus multiplication might sometimes go on in the cell wall that led me to suggest the earlier Symposium 'The Nature of the Bacterial Surface' and the point was raised in the Introduction. Bawden and I find (*Brit. J. Exp. Path.*, 1946, **27**, 81) that as much as three-quarters of the anomalous nucleoprotein in the leaf of a tobacco plant infected with tobacco mosaic virus may be anchored to the cell stroma. It is not released by proteases but is by enzyme preparations containing cellulase. Would you have any means of refuting the suggestion that influenza virus multiplies in the cell wall and then diffuses from it, partly into the body of the cell and partly into the surrounding fluid? I realize that there is no clear evidence for such a mode of multiplication but can it be excluded?

T. H. FLEWETT: The suggestion that viruses of the influenza group multiply in or near the cell wall cannot be refuted on the evidence of electron micrographs. But the fact that mature virus particles are not seen in the depths of the cytoplasm does not mean that virus is not multiplying there in a form which is less electron optically dense, and therefore undetectable in sections of tissue, than the virus as it is found in the allantoic cavity after liberation from the infected cells. The electron micrographs of influenza-infected cells suggest that if the surfaces of the cells could be stripped off, as Dr Bawden and Dr Pirie have been able to do with plant cells, most of the infectivity would be found in the surface. It does not follow that most of the virus antigen would also be in this fraction.

These remarks only apply to influenza and fowl plague viruses. Some other viruses, e.g. *Vaccinia*, *Molluscum contagiosum*, *Herpes febrilis*, do appear to grow all through the cell cytoplasm.

M. H. SALAMAN: I should like to describe an investigation of the development of psittacosis in chick embryonic tissues by methods somewhat similar to those used by Dr Flewett.

Dr Barwell and I, in Professor Bedson's Department at the London Hospital, have followed the development of the virus in living chick cells by phase contrast and ultra-violet light microscopy. In whole mounts of amniotic membranes from infected eggs, and in tissue cultures of amniotic epithelium and of fibroblasts infected in vitro, large spherical bodies, 1 to 3 μ in diameter, were first seen in the cytoplasm 13 to 17 hours after infection. They were difficult to detect at first, as their refractility was low, and their ultra-violet absorption very similar to that of mitochondria. They usually occurred in small clumps of 2 to 8, but were sometimes arranged in extended groups. Neither phase contrast nor ultra-violet revealed any containing envelope at this stage.

During the following 36 hours these bodies increased greatly in numbers and decreased in size, and formed definite colonies surrounded by well-defined envelopes. The enclosed bodies formed a tightly packed layer inside this envelope, but there often appeared to be empty spaces in the depths of the colony.

Up to about 24 hours after infection no general changes in the host cell were noted. Nucleus and nucleoli, and mitochondria, appeared healthy, even in cells containing two or more large colonies of virus occupying as much as half the cytoplasmic volume. The colonies did not appear to be in any fixed relation to any intra-cellular structure, nor to the cell membrane.

After this time, the colonies increased still further in size, and the enclosed virus bodies became smaller and more numerous and began active Brownian movement.

Meanwhile the nucleus and cytoplasm of the host cell became more and more transparent, until little but the cell membrane could be seen. Virus colonies often escaped from the tissue after about 48 hours, and floated about as compact spheres.

It will be noted that these observations correspond very closely to those made by Bedson and Bland on both stained and living preparations. Virus forms are detectable a little earlier in the former. The appearances in both, as Bedson and Bland pointed out, strongly suggest multiplication by binary fission from the time the virus first becomes visible.

For the purpose of the present discussion, I may summarize these observations as follows:

(1) The virus was first seen about 13 hours after infection as large spherical bodies, of low refractility and ultra-violet absorption. They were so inconspicuous at first that they may have been missed for some hours before this.

(2) These bodies increased in number and decreased in size during the following 36 hours, forming definite colonies, surrounded by an envelope, and becoming more refractile and absorptive of ultra-violet light. This development strongly suggests multiplication by binary fission.

(3) No alteration in the normal structures of the host cell was noted for at least 24 hours after infection.

THE ARGUMENT FOR A VENERABLE HYPOTHESIS OF VIRUS MULTIPLICATION

FORREST FULTON

London School of Hygiene and Tropical Medicine

INTRODUCTION

It is convenient to arrange those viruses which multiply inside the cells of animal tissues in a series according to size. Although the viruses have never been shown to possess enzymatic activity on simple substrates, yet it is difficult to believe that they possess no synthetic mechanisms, if only such as to enable them to assemble complex substances into their peculiar architecture. Therefore it is plausible to assume that such an arrangement according to size will group together elements which possess similar degrees of synthetic power. Because such basic synthetic powers are the consequence of the possession of discrete genes, and because the sizes of the smaller viruses are probably only small multiples of the sizes of the genes, those elements with the simplest synthetic power will also be the smallest.

We do not certainly know that the series is a simple one consisting of like elements, for it may be that we have arbitrarily arranged in a series a number of distinct groups. However, at present, no such discontinuities are apparent.

Many bacteria which multiply in animal tissues also prefer to multiply inside cells and it is, therefore, natural to suggest that the viruses are merely the end of a much larger series whose upper members comprise the bacteria (Green, 1935). This hypothesis is supported by the existence of the rickettsiae and the members of the psittacosis group which possess striking similarities both to bacteria and to viruses and seem to supply a natural link.

The larger series has the obvious discontinuity that at the point where the virus elements commence, the synthetic power of the organism has fallen below the threshold permitting independent existence. But the idea of the viruses as elements of a larger series has resulted in the hypothesis that viruses are small bacteria which possess some but not all their properties. In the absence of specific evidence it was further assumed that viruses, though forced to seek the inside of a suitable cell, multiply in the same way that bacteria multiply, by fission.

This assumption has recently been challenged as a consequence of experiments with bacteriophage. The idea that bacteriophages are viruses parasitic on bacteria and members of the same series as animal viruses has proved a very fruitful hypothesis. The detailed correspondences which have been shown to exist between the behaviour of some of the lytic phages and the influenza viruses have been so striking that there is a reasonable tendency to assume that facts discovered in experiments with bacterial viruses will be found to be applicable to animal viruses.

MULTIPLICATION OF BACTERIOPHAGE

Luria (1947) has suggested that lytic bacteriophages of the T series do not multiply by binary fission, but that on entering the bacterial cell they split up at once into small units which are the individual genes. The genes then multiply independently by redirecting the cell's enzymes to their own replication, and only later, about half-way through the development cycle, come together again to form a number of new phage particles.

This theory was put forward as a working hypothesis for which it was admitted there was no direct evidence. It was evolved to account for the very high efficiency of multiple infection with ultraviolet inactivated phage in giving viable phage. The inactive phage particles were imagined to have different essential genes which had been destroyed by the irradiation. If a bacterium was infected with a number of inactive particles, active phage could be produced provided that the intact gene units brought in by the particles in the aggregate made up a complete set. This recombination could have been supposed to take place by the reciprocal interchange of portions of the linkage groups. However, for the emergence of a particle with a complete set of undamaged genes several exchanges would have to be postulated, and only a few viable recombinants would have been expected. An alternative hypothesis in which the genes were supposed to multiply independently would provide a simple explanation of the phenomenon and of its efficiency, and would also be compatible with a number of other puzzling observations. It would explain the disappearance of the infective phage particle immediately after it has entered the bacterial cell, and also the non-clonal distribution of active phage recombinants. But because the independent multiplication of genes with their subsequent collection into new phage particles is such a novel idea, critical consideration is necessary.

Luria has stated (1950) that in the multiplication of bacteriophage the essential process must be the formation of replicas of the chemical configurations which embody the specificity of the organism, increase in size and division being epiphenomena. But this must also be true at the molecular level for any process of multiplication. The phenomena of mutation and recombination in phage, and the results of irradiation all seem to make it certain that the specificity of a phage particle is perpetuated by discrete sub-units, and these sub-units must be the units which are replicated. But all cells have their specificity perpetuated by discrete sub-units called genes. In animal and plant cells these genes are physically bound together into linkage groups called chromosomes, and their replication is not an isolated process but is integrated with the rest and is associated with fission of that linkage group.

The specificity of a complex cell is not due to the possession of a particular gene but is due to the fact that the cell possesses a particular collection of genes; and, in the cells of animal and plant tissues and probably bacteria, specificity is due to the possession of a particular collection of genes arranged in the linkage groups in a particular order. The number of genes which determines the specificity of a bacteriophage particle is unknown but is probably of the order of one hundred. If the individual genes of this collection break free from the others and multiply independently there must be some force to bring the specific collection together again: it would be necessary to postulate a vital force acting without a material counterpart. For no random reassortment could, with such large numbers of genes, result in the economical perpetuation of a specific bacteriophage particle. But so long as the genes are kept together in their linkage groups their persistence as a collection is assured.

Owing, therefore, to the difficulty of any alternative idea, the hypothesis that the genes are bound together into linkage groups is preferable until it has been disproved decisively. And this hypothesis implies multiplication by fission of the linkage groups. As in animal and plant cells fission of the linkage group may be followed by their random reassortment, perhaps as a consequence of the formation of a multinucleate cell. If, as seems likely, there is more than one type of linkage group, the phage may not only show recombination between homologous chromosomes but also various types of polyploids may result.

The multiplication of phage by a fission process has not yet been decisively disproved. For it is certain that the disappearance of the infective particle is not the same thing as the disappearance of the particle. It is possible to argue that the disappearance of the infective particle is an artefact due to the technical difficulty of recovery. More probably, however, the phenomenon is a real one caused by some change in the virus particle. But this change may be quite superficial resulting, perhaps, in the loss of specific surface receptors.

The non-clonal distribution of recombinants of active virus may be explained as a consequence of recombination occurring later, after multiplication has taken place. This seems a reasonable assumption since recombination of the K-12 strain of *Bact. coli* occurs under conditions in which large numbers of organisms are placed in a situation which favours pairing. It is, perhaps, significant that there is a clonal distribution of phage mutants. The arithmetical linear increase in phage particles can be explained in a number of ways which do not disprove fission, and indeed this type of increase has been observed during the multiplication of *Mycobacterium tuberculosis* in some kinds of fluid media (Fisher, Kirchheimer & Hess, 1951).

Recombination in itself does not preclude a fission process for multiplication of phage, though the non-reciprocal nature of genetic transfers cannot be explained on the available evidence. However, the same problem exists in bacterial recombination. The phenomenon of photoreactivation of phage inactivated by ultraviolet light (Dulbecco, 1950) has emphasized how little is known about the inactivation process. The inactivated particles seem still to possess a number of the activities of the fully active phage particles.

The measurement of DNA synthesis in bacteria multiply-infected with inactivated phage does not support the idea of a prior multiplication of genes, for the synthesis only occurs after a long lag period and just precedes the first infective particles (Cohen & Arbogast, 1950).

A plausible hypothesis might be that the phage inactivated by ultraviolet light is able to multiply, after a lag, by fission, but that recombination at a later stage is necessary to restore infectivity.

MULTIPLICATION OF INFLUENZA VIRUS

At the time when Luria's hypothesis of bacteriophage multiplication was first proposed, Hoyle (1948) suggested that influenza virus multiplies inside suitable cells in the same way.

The occasion which prompted this suggestion was the observation that complement fixing soluble antigen could be detected in infected egg tissues some hours before haemagglutinin, the latter only being known associated with the elementary bodies. Therefore, it was argued, the soluble antigen precedes the elementary body; therefore the soluble antigen is the precursor of the elementary body. The soluble antigen was known to be very much smaller in size than the elementary body so that it was natural to call the soluble antigen the tissue form of the virus particle and equate it with Luria's gene pool. But it has been shown that at the same time as the soluble antigen appears, the specific complement fixing antigen which is only known associated with the elementary body also appears (Fulton, 1949; Kirber & Henle, 1950). Recently Liu and Henle (Liu & Henle, 1951) have found that if the infected tissues are treated with RDE, haemagglutinin is demonstrable as early as complement fixing antigen, the apparent difference in time being due to the technical difficulty of demonstrating elementary bodies by haemagglutination when compared with their demonstration by complement fixation. Thus, from the experimental evidence there is no support for the belief that the soluble antigen precedes the elementary body in time. The two appear together and increase in step. It is true that it is possible to suppose that the conversion of soluble antigen into elementary bodies is so rapid that they only appear to arise simultaneously, but this is a transcendental idea without any empirical foundation.

Despite the dazzling successes which have stemmed from the hypothesis that phage is a member of the virus series, yet this hypothesis should not mistakenly be supposed a proven fact. On the available evidence of lysogenicity it would be possible to state a case, however intuitively disagreeable, in support of an alternative hypothesis that phage is not a parasite at all, but plays some obscure role in the bacterial economy, and so is better grouped with the cytogenes and transforming substances.

Because of this doubt as to the true nature of phage, any fact discovered in experiments with phage cannot be simply transferred to animal viruses without independent proof.

MULTIPLICATION OF BACTERIA

Under optimal conditions of cultivation in the laboratory it is certain that the multiplication of bacteria is by binary fission. In animal and plant cells multiplication is also by fission, and in these cases the process may be divided into two distinct phases, the fission of the nucleus followed by the fission of the cytoplasm. The same is very probably true also of bacterial fission, the primary happening being, in every case, fission of the linkage groups. To assert that the linkage groups undergo binary fission is not to imply any fundamental understanding of the mode of replication of the genes composing the linkage group. The process of replication, whatever that may be, is believed to occur in such a way that the new strand of daughter genes lies, at its inception, in very close proximity to the original linkage group, like genes in the two strands lying immediately adjacent. Later the two strands will move apart. This process will appear to the observer to be a thickening of the linkage group, followed by its fission into two strands.

Under certain conditions the two phases of fission may not follow one another in a regular sequence, so that the first phase may proceed at a higher rate than the second and the second may not occur at all. In the latter case there cannot strictly be said to be multiplication since new daughter cells have not originated from the one parent; but multiplication may occur later by the fragmentation of the multinucleated filaments or spore cysts which have resulted from the fission of the linkage groups. The L-forms of bacteria may be a type of multinucleated spore cyst.

Thus, to believe that viruses multiply as if they were small bacteria does not necessarily imply a belief in binary fission, though that is the simplest hypothesis; but it does imply a belief in the binary fission of the linkage groups. It requires a certain integrity of the virus units so that their identity is at all times distinct from that of the host. If it should prove that in order to utilize the enzymes and complex labile substances of the host cell, the individual genes must part company and seek out their special zone in the cytoplasm, only later to come together to form new elementary bodies (Bauer, 1949), then it would have to be admitted that viruses do not multiply like small bacteria. But there is no evidence that such drastic fragmentation in fact occurs.

VIRUS INCLUSION BODIES

The cause of the present division of opinion as to how viruses multiply is the lack of direct evidence. Among the rickettsiae and the members of the psittacosis-lymphogranuloma group which seem to provide a natural link between viruses and bacteria, it is possible, owing to their size, to attempt direct visual microscopical examination of their modes of multiplication. *Rickettsia mooseri* adapted to grow in the lungs of mice may be studied after intra-nasal instillation by sacrificing mice at intervals and examining impression smears. Soon after infection a few cells may be observed with a single rickettsia in the cytoplasm. Later this single rickettsia appears to grow in size, and later still it is possible to resolve the morula into a cluster of tiny rickettsiae which increase in size and finally break out of their nest (Begg, Fulton & van den Ende, 1944). Observations of psittacosis strains growing in mouse lung or on the chorio-allantoic membrane of chick embryos disclose a similar process. The point of uncertainty is what exactly is happening between the settling down of the single elementary body and the appearance of the distinct morula. Two hypotheses seem plausible. Either the growth observed is due to the binary fission of the original particle, but owing to the close packing of the daughter elementary bodies they cannot be optically resolved until they have increased in size; or the initial period is confined to a multiplication of the linkage groups in a kind of spore cyst which later, at the time the morula becomes visible, fragments into daughter elementary bodies.

With the true viruses the direct evidence is even more equivocal, though it is reasonable to hope that this is only a transitional state of affairs and that eventually electron microscopy will provide indisputable proof.

In some virus infections it is possible to find, at a late stage when much multiplication has taken place, distinct inclusion bodies. It is very probable that these inclusion bodies are colonies of virus elementary bodies exactly analogous to the morulae of the rickettsiae and the psittacosis group.

In other virus infections inclusion bodies have not been demonstrated. The failure to find them may be due either to their not being there to find or their not being demonstrable by the techniques used.

It is probable that the virus nests are only easily seen when they provoke a certain grade of reaction from the cytoplasm around them, though when such a reaction does not occur there is no need to suppose that the multiplying elementary bodies are strictly confined to a nest.

In the case of cells infected with the influenza virus and with some other viruses of the same group, inclusion bodies have not been demonstrated, but instead, electron photomicrographs show that, late in the development cycle, the cytoplasm of the cell contains many long filaments. (Murphy, Karzon & Bang, 1950; Flewett & Challice, 1951). These filaments have a cross-section which is equal to the diameter of the elementary body, and it is reasonably certain that elementary bodies can be, and perhaps are always, derived from the fragmentation of these filaments (Chu, Dawson & Elford, 1949).

It is a working hypothesis, not contradicted by any known fact, that the filaments were each derived from one elementary body by a process of fission. It is impossible to decide the exact nature of the filament for it could either be multinucleate or be merely a string of elementary bodies adhering together in the plane of fission. Some electron photomicrographs show the virus filaments actually inside cells, others (Morgan & Wyckoff, 1950) suggest that the virus may be multiplying not inside but on the surface of cells, in the same way that members of the psittacosis group are said sometimes to grow (Weiss, 1949). However, this apparent surface multiplication of virus is seen in preparations in which cell necrosis has already begun, so that it seems more probable that the virus filaments are merely entangled in shreds of protoplasm from cells which are beginning to break down.

Direct observation of bacteria infected with bacteriophage has not yet provided convincing direct evidence of the way phage multiplies, but there is one fact which seems to be rather different from that observed with animal viruses. When a lytic phage particle enters a bacterial cell the evidence suggests that the nucleus of that cell is immediately disrupted and that the fragments are scattered to the periphery. The phage seems to usurp the place of the cell's own nucleus so that the phage-infected bacterium is a different entity under wholly new management. The cells of animal tissues infected with a virus are not so completely dominated. When the virus settles down in the cytoplasm or in the nucleus of the cell, maintenance of the structure of the cell seems to be an essential pre-requisite for virus multiplication. For if the cell is destroyed there is nothing for the virus to plunder. It is possible that this difference between the behaviour of the lytic phages and the animal viruses is not a fundamental one but is related to the relatively very much faster growth cycle of the phage.

Although phage seems only able to get out of a bacterial cell by bursting it, there is no certain knowledge if this is the only way by which animal viruses can get out of cells. It is possible that they may, as has been suggested by Hoyle emerge encased in a protoplasmic thread extruded from the main body of cytoplasm, complete freedom then depending upon break up of the protoplasmic thread.

SOLUBLE ANTIGENS ASSOCIATED WITH VIRUSES

In tissues in which viruses have multiplied it is usual to find soluble antigens specific for the virus concerned, the same soluble antigen being found in the tissue of quite different hosts. They have been described associated with the Pox group of viruses, Rabies virus, Herpes simplex virus, viruses of the Mumps/Influenza group and with the virus of Lymphocytic Choriomeningitis. They have also been found associated with rickettsiae, with members of the psittacosis-lymphogranuloma group and with bacteriophage (Burnet, 1933). These soluble antigens are, as their name implies, much more highly dispersed than the elementary bodies of the virus and they can be detected by the complement fixation technique.

When bacteria grow in a tube of broth they not only multiply but they add to the broth substances which diffuse away from them. Some of these substances may be antigenic and a part of the antigenic material will have been derived from the antigens which, at their inception, occupied a position on the surface of the bacterium. It is a working hypothesis that the soluble antigens of viruses are produced in the same way.

It is known with certainty that this hypothesis is correct in the case of the soluble antigen of *Rickettsia prowazeki.* If tissues, in which the rickettsiae have multiplied, are ground in a mortar the antigenic material can be divided by centrifugation into two main parts. One part contains the rickettsial bodies, the other part contains an antigen of much smaller particle size which is the soluble antigen. This soluble antigen is exactly analogous to [PLAPS] of Brucella (Miles & Pirie, 1939). When a purified suspension of rickettsiae is shaken with ethyl ether it can be shown by electron microscopy that fragments of the surface capsule are torn off (Shepard & Wyckoff, 1946). These fragments may be separated from the rickettsial bodies by centrifugation and they behave in every way like soluble antigen. It is true that without purification and exact chemical identification it is impossible to be certain that the soluble antigen derived in this way from the rickettsial bodies is identical with that found associated with the developing rickettsiae in the tissues: however, the two dispersed antigens behave serologically in a similar manner and it is assumed that they are also similar chemically.

Two species of rickettsiae, *R. prowazeki* and *R. mooseri* can be differentiated antigenically by their capsular material, but when this material is torn off the rickettsial bodies with ethyl ether the resulting soluble antigens are indistinguishable; the process of removal from the surface has blunted their specificity (Fulton & Begg, 1946). These soluble antigens may be further degraded by boiling and when the boiled antigens are injected intravenously into rabbits they evoke antibodies which not only react with the boiled soluble antigens but also with some component on the surface of Proteus OX 19. Crude rickettsial suspensions injected intravenously into rabbits evoke a whole range of antibodies some of which react with the undamaged antigen on the surface of the rickettsiae, others reacting with various degradations of these antigens and with Proteus OX 19. In contrast, rabbits injected intravenously with purified washed rickettsial bodies which have been fixed by the addition of formalin, develop an antibody spectrum which is much simpler, the antibodies being confined to the more specific fraction and the Weil-Felix reaction is in consequence negative.

The soluble antigen of the psittacosis virus (Bedson, 1936) behaves in very much the same way, the slight apparent differences may plausibly be said to stem from the use of unpurified elementary body suspensions in adsorption experiments, and the more labile nature of the surface antigen.

With these examples in mind it is natural to assume that the soluble antigens associated with the true viruses are of the same nature. The soluble antigen of the vaccinia virus is certainly derived from the surface antigen of the elementary bodies and this soluble antigen can, like that of the rickettsiae, undergo a sequence of antigenic degradations (Craigie & Wishart, 1936). Of the other virus soluble antigens the only one for which there is an adequate body of information is the soluble antigen of the influenza virus. Its properties (Wiener, Henle & Henle, 1946) show remarkable similarity to the rickettsial soluble antigens. Thus it is serologically less specific than the elementary body; for the A strains are readily distinguished from the A′ strains by the antigenic structure of the elementary bodies, but the soluble antigens of the two groups are apparently identical. Influenzal soluble antigens may be obtained by shaking purified elementary bodies with ether. Patients who are infected with influenza virus develop antibodies both for the soluble antigen and for the elementary body; whereas those inoculated with vaccine prepared from washed elementary bodies preserved with formalin develop antibodies only for the elementary bodies. It is true that this fact may also be explained by the hypothesis that the soluble antigen is a tissue form of the influenza virus, but with rickettsia, when the same facts hold, we know that such an explanation is not correct.

Two simple hypotheses which explain the facts seem worthy of consideration. The soluble antigen is exactly analogous to the soluble antigen of the rickettsiae and represents a dispersed and degraded form of the surface antigen of the elementary bodies, the antigen being no longer capable of adsorption on to the receptors of chicken erythrocytes.

Alternatively, the soluble antigen has nothing whatever to do with the antigen of the elementary bodies. It might be supposed to be a matrix substance similar to that making up the nest of the inclusion body of the Molluscum contagiosum virus. The matrix substance might be regarded as a ground substance of a spore cyst in which the linkage groups of the infecting virus particle are multiplying by binary fission.

It has been observed that some bacteriophages of the T series, when growing on their bacterial host in a lactate medium, have a coating of desoxyribosenucleic acid (Cohen, 1947). This coating may be removed with desoxyribonuclease without affecting the infectivity of the phage particle. It is possible that this observation provides a clue as to the true nature of the soluble antigen of influenza virus.

INFECTIVE AND NON-INFECTIVE VIRUS

One of the main intuitive supports for the belief in a complex growth cycle for virus multiplication rests on the apparent existence of non-infective virus particles. If a bacterium is infected with an active lytic phage particle and if the bacterium is artificially broken open (Doermann, 1951) at some time in the development cycle of the phage before natural lysis would have occurred, it has been shown that active phage cannot be obtained immediately after infection nor until about half-way through the growth cycle. After infection a lag occurs and then DNA synthesis commences. Some minutes after this, infective phage particles may be obtained. Within the interval between commencement of DNA synthesis and the appearance of infective phage, the phage particle may be demonstrated by complement fixation (Rountree, 1951).

In just the same way, influenza virus deposited on the chorio-allantoic membrane of a developing chick embryo is immediately taken up by the cells lining the membrane. But if the membrane is then ground in a mortar, the infective virus particles cannot be recovered. Infective virus appears again after a lag period and, as in the case of the phage, the presence of the particle may be demonstrated by complement fixation before it can be demonstrated by infectivity.

The problem may be analysed by considering what happens to the phage particle when it first gets inside a cell, and what is the nature of the virus particle which can be demonstrated by complement fixation but not by infectivity.

To assume that because an infective particle can no longer be recovered as an infective particle, that the particle itself has disappeared, is an unwarrantable assumption. Disregarding the difficulty of getting a virus particle out of a cell and free from thwarting entanglements, it has just fought its way through a cell wall by an enzymatic process and it may be in no fit state to do it again. Provided that it is not merely a technical difficulty of recovery, the simplest hypothesis is that the surface of the particle has changed.

The second part of the problem is rather different. The bacteriophage particle which is detected in the interval between commencement of DNA synthesis and appearance of infective phage, seems to have the same surface antigenic structure as the final phage particle, for both react with the same antibody. In the case of the influenza virus, the actual surface antigen of the intact virus particle is being measured. There is no reason to suppose that the specific antigen is in a highly dispersed state and it may reasonably be supposed to occupy a position on the surface of a particle, as it does in the fully infective virus.

The question arises why the virus particles are not immediately infective. Two alternative explanations are suggested. The first hypothesis supposes that the particles are infective, but that because of their aggregation no rise of infectivity can be shown until dispersion has commenced. In the specific instance of the influenza virus, assuming that the long filaments are strings of elementary bodies, infective virus cannot be demonstrated until the long filaments have begun to fragment into discrete elementary particles. In support of this view there is an anomalous finding recorded by the Henles (Henle & Henle, 1949) who, under certain conditions could demonstrate a rise of infectivity before a rise of haemagglutinin, which is the reverse of the usual finding. The conditions of this anomalous result were such as to make the infectivity test more sensitive by neutralizing that part of the infective seed which had remained extracellular.

The second hypothesis would accept the fact that the virus particles are at first non-infective, some maturation process being necessary. As the surface antigen of the postulated precursor particle and of the infective particle appear to be similar, it must be supposed that the maturation involves a change more fundamental than a surface modification or more subtle than the serological methods of identification. With this hypothesis the elementary virus particle capable of infecting a suitable cell might be regarded as analogous to a bacterial spore, and the elementary virus particle multiplying inside the cell as the vegetative form. In the specific instance of the influenza virus, assuming the long filaments to be multinucleated cells, these are the vegetative forms from which the spore forms develop by fragmentation into uninucleated elementary bodies.

The main support for the maturation hypothesis is contained in the work of von Magnus (1951). He has shown conclusively that if the allantoic sac of a chick embryo is infected with a gross excess of infective influenza virus particles, the resulting yield of virus consists mainly of particles which are identical in most respects to the parent particles except that they are non-infective. Both seem to have similar surface antigens, and both cause haemagglutination. They have, by filtration, the same size, though the non-infective particles sediment in centrifugal fields rather differently. The interpretation has been that the overworked host cells have become weary and have turned out half completed virus particles. This interpretation necessarily implies that normally the particle goes through a process of maturation before it achieves its crowning attribute of power to infect suitable cells.

The explanation is certainly plausible and cannot be refuted at present, but it seems to be only one of a number of possible hypotheses. Perhaps multiple infection of the cell has, by recombination, resulted in a new genotype which evokes a pheno-typically different virus particle. Boyd has suggested (1951) that the phenomenon may be in some way related to the analogous behaviour of some lytic phages which undergo a diphasic variation to a symbiotic form. Perhaps the explanation is that the virus clone is heterogeneous as a consequence of mutation and recombination and that infection with a gross excess of virus results in the selection of a fraction of the total population with a lower infectivity. Specifically, it may be argued that the particles with a low infectivity possess too slow a growth rate to yield a new crop of daughter particles, or alternatively that their genotype is defective and results in a phenotype unable to infect. Some of the results of growing influenza virus in eggs kept at temperatures above 36° are very curious and have apparently resulted in virus clones which are infective but not haemagglutinating (Blumenthal, Greiff, Pinkerton & DeWitt, 1950).

CONCLUSIONS

It is admitted that, at present, the direct evidence for the mechanism of virus multiplication is so scanty, and the indirect evidence is so debatable that there is ample ground for disagreement. In these circumstances vanity enjoins caution in the explicit expression of beliefs. However, the firm statement of a working hypothesis which can be refuted and emended in detail is so essential for progress in understanding that an explicit statement will be attempted.

It is supposed that viruses possess genes arranged in linkage groups and that the number of different kinds of linkage groups in a virus particle is a small number but greater than one.

These linkage groups multiply by binary fission and, in consequence, the mode of multiplication of viruses is, in essence, no different from the multiplication of bacteria or indeed of any other cell.

The simplest hypothesis is to suppose that the phage particles also multiply by binary fission in step with fission of the linkage groups, but the formation of multinucleated cells is a distinct possibility. Recombination is a very common occurrence and it normally takes place late in the cycle, after multiplication of the virus. Assuming binary fission of the phage particles, it is supposed that the recombination occurs by fusion of two or more phage particles followed by segregation in which polyploid particles of various types may result. If multinucleated cells are formed it is possible that in some cases they arise by fusion of several infecting units, with later recombination among the linkage groups.

Recombination together with point mutation is the cause of the phenotypic variation among virus particles. It is believed that the appearance of the non-infective particles is, in part, an illusion due to aggregation of the particles immediately following fission, but is probably also in part a real phenomenon due to great differences in the genotype of the particles as a consequence of recombination and mutation.

It is supposed that the soluble antigens associated with viruses have a dual origin. They are derived from the surface antigens of the particle by fragmentation and degradation; and they represent a matrix substance surrounding the multiplying virus, formed as a direct consequence of the active synthesis of specific virus material, not all of which is built into the final particle.

REFERENCES

Bauer, D. J. (1949). Multiplication of the animal viruses. *Nature*, **164**, 767.

Bedson, S. P. (1936). Observations bearing on the antigenic composition of psittacosis virus. *Brit. J. Exp. Path.*, **17**, 109.

Begg, A. M., Fulton, F. & van den Ende, M. (1944). Inclusion bodies in association with typhus rickettsiae. *J. Path. Bact.*, **56**, 109.

Blumenthal, H. T., Greiff, D., Pinkerton, H. & DeWitt, R. (1950). Influenza. I. The haemagglutination and infectivity titre curves of PR8 influenza virus cultivated in embryonated eggs at different temperatures. *J. Exp. Med.*, **91**, 321.

Boyd, J. S. K. (1951). 'Excessive dose' phenomenon in virus infections. *Nature*, **167**, 1061.

Burnet, F. M. (1933). A specific soluble substance for bacteriophages. *Brit. J. Exp. Path.*, **14**, 100.

Chu, C. M., Dawson, I. M. & Elford, W. J. (1949). Filamentous forms associated with newly isolated influenza virus. *Lancet*, 1, 602.

Cohen, S. S. (1947). Streptomycin and desoxyribonuclease in the study of variations in the properties of a bacterial virus. *J. Biol. Chem.*, **168**, 511.

Cohen, S. S. & Arbogast, R. (1950). Chemical studies on host-virus interactions. VIII. The mutual reactivation of T_2r+ virus inactivated by ultraviolet light in the synthesis of desoxyribose nucleic acid. *J. Exp. Med.*, **91**, 637.

Craigie, J. & Wishart, F. O. (1936). Studies on the soluble precipitable substances of Vaccinia. I. The dissociation *in vitro* of soluble precipitable substances from elementary bodies of vaccinia. *J. Exp. Med.*, **64**, 803.

Doermann, A. H. (1951). Intracellular phage growth as studied by premature lysis. *Fed. Proc.*, **10**, 591.

Dulbecco, R. (1950). Experiments on photoreactivation of bacteriophages inactivated with ultraviolet radiation. *J. Bact.*, **59**, 329.

Fisher, M. W., Kirchheimer, W. F. & Hess, A. R. (1951). The arithmetic linear growth of *Myco. tuberculosis* var hominis. *J. Bact.*, **62**, 319.

Flewett, T. H. & Challice, C. E. (1951). The intracellular growth of fowl-plague virus. *J. Gen. Microbiol.*, **5**, 279.

Fulton, F. (1949). Growth-cycle of Influenza Virus. *Nature*, **164**, 189.

Fulton, F. & Begg, A. M. (1946). The antigenic structure of typhus rickettsiae. *Spec. Rep. Ser.: Med. Res. Council, London*, No. 255, p. 163.

Green, R. G. (1935). On the nature of filterable viruses. *Science*, **82**, 443.

Henle, W. & Henle, G. (1949). Studies on host-virus interactions in the chick embryo-influenza virus system. III. Development of infectivity, haemagglutination and complement fixation activities during the first infectious cycle. *J. Exp. Med.*, **90**, 23.

HOYLE, L. (1948). The growth cycle of influenza A virus. A study of the relations between virus, soluble antigen and host cell in fertile eggs inoculated with influenza virus. *Brit. J. Exp. Path.*, **29**, 390.

HOYLE, L. (1950). The multiplication of influenza viruses in the fertile egg. *J. Hyg.*, **48**, 277.

KIRBER, M. W. & HENLE, W. (1950). A comparison of influenza complement fixing antigens derived from allantoic fluids and membranes. *J. Immunol.*, **65**, 229.

LIU, O. C. & HENLE, W. (1951). Studies on host-virus interactions in the chick embryo-influenza virus system. IV. The role of inhibitors of haemagglutination in the evaluation of viral multiplication. *J. Exp. Med.*, **94**, 269.

LURIA, S. E. (1947). Reactivation of irradiated bacteriophage by transfer of self-reproducing units. *Proc. Nat. Acad. Sci., Wash.*, **33**, 253.

LURIA, S. E. (1950). Bacteriophage: an essay on virus reproduction. *Science*, **111**, 507.

VON MAGNUS, P. (1951). Propagation of the PR8 strain of influenza virus in chick embryos. II. Formation of 'incomplete' virus following inoculation of large doses of seed virus. *Acta path. microbiol. Scand.*, **28**, 278.

MILES, A. A. & PIRIE, N. W. (1939). The properties of antigenic preparations from *Brucella melitensis*. I. Chemical and Physical properties of bacterial fractions. *Brit. J. Exp. Path.*, **20**, 83.

MORGAN, C. & WYCKOFF, R. W. G. (1950). The electron microscopy of fowl pox virus within the chorio-allantoic membrane. *J. Immunol.*, **65**, 285.

MURPHY, J. S., KARZON, D. T. & BANG, F. B. (1950). Studies of influenza virus A (PR8) infected tissue cultures by electron microscpoy. *Proc. Soc. Exp. Biol., N. Y.*, **73**, 596.

ROUNTREE, P. M. (1951). Complement fixing antigen of *Bact. coli* bacteriophage T_5: its behaviour during virus growth. *Brit. J. Exp. Path.*, **32**, 341.

SHEPARD, C. C. & WYCKOFF, R. W. G. (1946). The Nature of the Soluble Antigen from typhus rickettsiae. *Publ. Health Rep. Wash.*, **61**, 761.

WEISS, E. (1949). The extracellular development of agents of the psittacosis-lymphogranuloma group (Chlamydozoaceae). *J. Infect. Dis.*, **84**, 125.

WIENER, M., HENLE, W. & HENLE, G. (1946). Studies on the complement fixing antigens of influenza viruses A and B. *J. Exp. Med.*, **83**, 259.

THE MULTIPLICATION OF INSECT VIRUSES*

G. H. BERGOLD

Laboratory of Insect Pathology, Department of Agriculture, Canada

The insect viruses occupy a unique place among all other viruses with respect to the degree of purity with which they can be obtained. This fortunate circumstance is due to the fact that the virus particles are enclosed in inclusion bodies from which they can be liberated easily. The inclusion bodies are water insoluble and resistant to all processes occurring during bacterial decomposition of insect larvae. They can be obtained, therefore, by simple centrifugation in gram quantities as a white, dry powder, free from any foreign contamination (Fig. 1). They can be stored dry or wet for several years without much change.

These inclusion bodies consist of about 95% of a homogeneous protein with a molecular weight from about 275,000 to 375,000, depending on the insect species, and of about 3 to 5% of virus particles (Bergold, 1947). The inclusion bodies are often surrounded by a thin membrane of identical or very similar composition to the polyhedral protein (Wyatt, 1950). The virus particles can be liberated by dissolution of the inclusion bodies in weak alkali (Fig. 2) and separated from the inclusion body protein by centrifugation. They do not give any serological cross-reaction with host material, which proves their high degree of purity (Bergold & Friedrich-Freksa, 1947).

There is another exceptional character in the insect viruses. The inclusion bodies containing the virus particles are in their early stage of development apparently of a liquid consistency. Later the concentration of the inclusion body protein becomes so high that aggregation and crystallization occur fixing rigidly all possible stages of development or multiplication of the virus particles. This fortunate fact, together with the high degree of purity of the virus preparations, encourages morphological studies and may justify the interpretation of the forms seen as steps in a developmental and multiplication cycle.

In a previous paper (Bergold, 1950) it was demonstrated that the virus particles are not homogeneous in size and shape. A developmental cycle and various multiplication forms were described for four different insect viruses. Investigations of other insect viruses led to the same conclusions and further research confirmed the presence of sub-units in the virus rods (Bergold, 1952*b*) that had been postulated earlier (Bergold, 1950).

---

* Contribution No. 53, Division of Forest Biology, Science Service Dept. of Agriculture, Ottawa.

At present three different groups of insect viruses can be distinguished:

1. The classical polyhedral viruses (genus* *Bollea* Bergold; Steinhaus 1949; Bergold 1952*a*), representing rod-shaped viruses occurring in great numbers in polyhedral-shaped inclusion bodies (polyhedral bodies) of 0·5 to 15 micron diameter.
2. Capsule (granulosis) viruses (genus *Bergoldia* Steinhaus; Steinhaus 1949; Bergold 1952*a*), representing rod-shaped viruses occurring singly in oval-shaped inclusion bodies (capsules, granules) of less than 1 micron length.
3. Spherical viruses (genus *Smithia* Bergold; Bergold 1952*a*), occurring in great numbers in polyhedral-shaped inclusion bodies (polyhedral bodies) of 0·5 to 15 micron diameter.

The life cycle and kind of multiplication are similar in the classical polyhedral and the capsule viruses. In both groups the individual virus particles develop within the developmental membrane from a spherical body with a diameter of about 20–40 millimicrons, to one or several rod-shaped particles with dimensions of about 20–27 × 200–400 millimicrons.

In the polyhedral viruses the majority of virus particles included in the polyhedral bodies are in the rod-shaped stage, which is the 'resting stage' for this group. The spherical developing stages are present only in small numbers. The rods often still carry the developmental membrane. In some viruses of this group, for example, the virus of *Bombyx mori* L. (silkworm), usually one or occasionally two rods develop within the developmental membrane (Fig. 3). In others, for example, the virus of *Porthetria dispar* L. (gypsy moth), four or more rods develop in the developmental membrane in a bundle arrangement (Bergold, 1948*a*) (Figs. 4, 5, 6). This is not a result of lengthwise splitting of one large rod but division takes place within the early spherical stage, in which individual small particles can be seen. Each of these particles develops into a rod. The mode of multiplication herein described postulates the development of only 2–5 virus rods from one spherical particle. The individual virus rods are surrounded by another, the intimate membrane, reminiscent of the membranes around bacteriophage described by Anderson (1949). One slender projection about 10 millimicrons in diameter and about 60 millimicrons long can often be observed at one end of the rods (Bergold, 1952*a*, 1952*b*).

---

* The general use of a binominal system for viruses is at present under discussion (Andrewes, 1951).

In addition to this rather low rate of multiplication another more efficient one seems to take place. Alkaline conditions not only liberate the virus particles from the polyhedral bodies, but also cause a disintegration of the virus rods (Bergold, 1952*b*). Small spheres of about the same diameter as that of the rods slip out usually at the ends of the intimate membrane (Figs. 4, 5, 7, 8). The number of these spherical subunits has not been determined; however, six or more may be present. The disintegration of the rods is, under natural conditions, caused not only by alkali. Other processes seemed to be involved (Fig. 9) since the characteristic, empty, intimate membrane has been demonstrated in virus-infected blood-cells of *Bombyx mori* L. before polyhedral formation, without any alkali treatment (Bergold, 1952). This indicates further that the complete multiplication cycle—sphere, rod, spheres—takes place in the cell nuclei and is finally arrested by the formation of the polyhedral bodies. Similar conclusions are drawn by Bird (1952), who observed various developing forms of a polyhedral virus in gut cells of *Diprion hercyniae* Htg. (European Spruce Sawfly).

The life and multiplication cycles of the second group, the capsule virus, are not so well known. In principle they seem to be very similar. From the spherical stage within the developmental membrane, there develops usually a single rod, or occasionally a double rod. Each single or double rod is surrounded by the oval-shaped inclusion body, the so-called capsule (Fig. 10) (Bergold, 1948*b*) or granule (Steinhaus, Hughes & Wasser, 1949). Occasionally several rods become enclosed forming larger inclusion bodies of varying shape. The majority of the virus particles enclosed in the capsules are in the rod-shaped stage which is the 'resting stage' for this group of insect viruses also. Only a few are present in the spherical developing stages (Fig. 11) (Bergold, 1950).

The rods are usually slightly curved and since they do not shed their developmental membrane readily it gives them a wider appearance. They are much more resistant to alkali and the disintegration of the rods to spheres has been observed only occasionally (Fig. 12). The rods in this group also show the slender projection at one end (Bergold, 1952*b*).

There is very little known yet about the third group of spherical viruses which was discovered only recently (Smith & Wyckoff, 1950, 1951). These viruses occur in great numbers in polyhedral-shaped inclusion bodies. Liberation of the virus particles by alkali shows that by far the majority are spherical. This stage seems to be the 'resting stage' in this group. It has been stated that no rods can be found (Smith & Wyckoff, 1950, 1951). In further investigations, rods and filamentous forms were demonstrated (Fig. 13) (Bergold, 1952*b*). It is, therefore, possible that the development and multiplication cycles of the spherical viruses are similar to those of the rod-shaped ones. The slender projection was also seen on some spheres, giving these insect viruses an appearance almost identical with bacteriophages (Bergold, 1952*b*).

DISCUSSION

It must be admitted that the foregoing interpretations of morphological studies are subject to criticism. The main problem is that it is difficult or impossible to investigate the dynamic process of multiplication by rather static observations (Luria, 1950). Yet it is felt that the insect viruses are an exceptional subject due to the fixation of the virus particles by formation of the inclusion bodies during the process of their development. Moreover, the dimensions of the virus particles necessitate the use of the electron microscope for morphological studies. This means that all observations are made on dry objects. Yet the quality and excellent reproducibility of the virus pictures seem to justify the method employed.

Another objection which might be raised is the use of alkali. In nature the inclusion bodies are carried into the mid gut with the food. There they are dissolved readily by the strong alkaline reaction (up to pH 10·0) of the gut juice and the virus particles are liberated. Yet it was shown by intra-lymphal infection tests that virus suspensions prepared with gut juice were much less infectious than those prepared with pure alkali. Therefore, serious destruction of the virus particles does not occur by the use of alkali.

A more important criticism is based on the fact that virus preparations treated with weak alkali in order to release the spherical sub-units had a decreased infectious activity (Bergold, 1952*b*). If the spherical virus particles are really not active, strong evidence against the validity of the multiplication cycle would be shown. Yet the inactivity could be explained in two other ways: the spherical stage may be very delicate and be inactivated during the treatment; or the spheres may not become absorbed by the host cells and therefore, may not arrive at the place of development and multiplication. Similar inactive phases during the early development steps are known in bacteriophage and other viruses (Bawden & Pirie, 1945; Gard & Magnus, 1946; Henle, Henle & Rosenberg, 1947; Hoyle, 1948; Heinmets & Golub, 1948; Markham, Matthews & Smith, 1948; Foster, 1948; Henle & Henle, 1949; Horsfall, 1949).

Working with rod-shaped polyhedral viruses, Smith and Wyckoff (1950, 1951) were unable to find the spherical stages. Yet the few experimental data given indicate the inadequacy of their method of virus preparation. In the meantime other investigators (Wasser & Steinhaus, 1951; Hughes & Thompson, 1951; Bird, 1952) confirmed the presence of some developing stages. Moreover, it is interesting to note that various developing stages, spherical forms together with rod-shaped and filamentous forms, have been described in other viruses too (Bedson, 1933; Tang & Wei, 1937; Rake & Jones, 1942; Bang, 1948; Heinmets & Golub, 1948; Horsfall, 1949; Hoyle, 1950; Putnam, 1950; Wyckoff, 1951; Brakke, Black & Wyckoff, 1951).

REFERENCES

ANDERSON, T. F. (1949). The reaction of bacterial viruses with their host cells. *Bot. Rev.*, **15,** 464.

ANDREWES, C. H. (1951). Viruses and Linnaeus. *Acta. path. microbiol. Scand.*, **28,** 211.

BANG, F. B. (1948). Studies on newcastle disease virus. III. Characters of the virus itself with particular reference to electron microscopy. *J. Exp. Med.*, **88,** 251.

BAWDEN, F. C. & PIRIE, N. W. (1945). The separation and properties of tobacco mosaic virus in different states of aggregation. *Brit. J. Exp. Path.*, **26,** 294.

BEDSON, S. P. (1933). Observations on the developmental forms of psittacosis virus. *Brit. J. Exp. Path.* **14,** 267.

BERGOLD, G. H. (1947). Die Isolierung des Polyeder-Virus und die Natur der Polyeder. *Z. Naturforsch.*, **2b,** 122.

BERGOLD, G. H. (1948*a*). Bündelförmige Ordnung von Polyederviren. *Z. Naturforsch.*, **3b,** 25.

BERGOLD, G. H. (1948b). Über die Kapselvirus-Krankheit. *Z. Naturforsch.*, **3b,** 338.

BERGOLD, G. H. (1950). The multiplication of insect viruses as organisms. *Can. J. Res.*, E, **28,** 5.

BERGOLD, G. H. (1952). Demonstration of polyhedral virus in blood-cells of silkworms. *Biochim. Biophys, Acta*, **8,** 397.

BERGOLD, G. H. (1952*a*). Concerning the nomenclature and classification of insect viruses. *Ann. New York Acad. Sci.* (in the press).

BERGOLD, G. H. (1952*b*). The morphology of insect viruses. (In preparation.)

BERGOLD, G. H. & FRIEDRICH-FREKSA, H. (1947). Zur Grösse und Serology des Bombyx-mori-Polyedervirus. *Z. Naturforsch.*, **2b,** 410.

BIRD, F. T. (1952). On the multiplication of an insect virus. *Biochim. Biophys. Acta.* **8,** 360.

BRAKKE, M. K., BLACK, L. M. & WYCKOFF, R. W. G. (1951). The sedimentation rate of potato yellow-dwarf virus. *Amer. J. Bot.*, **38,** 332.

FOSTER, RUTH A. G. (1948). An analysis of the action of proflavine on bacteriophage growth. *J. Bact.*, **56,** 795.

GARD, S. & VON MAGNUS, P. (1946). Studies on interference in experimental influenza. II. Purification and centrifugation experiments. *Ark. Kemi Min. Geol.*, **24 B,** No. 8.

HEINMETS, F. & GOLUB, O. J. (1948). Observations on the growth of psittacosis virus in chorioallantoic membranes by electron microscope. *J. Bact.*, **56,** 509.

HENLE, W. & HENLE, G. (1949). Studies on host-virus interactions in the chick embryo-influenza virus system. III. Development of infectivity, haemagglutination and complement fixation activities during the first infectious cycle. *J. Exp. Med.*, **90,** 23.

HENLE, W., HENLE, G. & ROSENBERG, E. B. (1947). The demonstration of one-step growth curves of influenza viruses through the blocking effect of irradiated virus on further infection. *J. Exp. Med.*, **86,** 423.

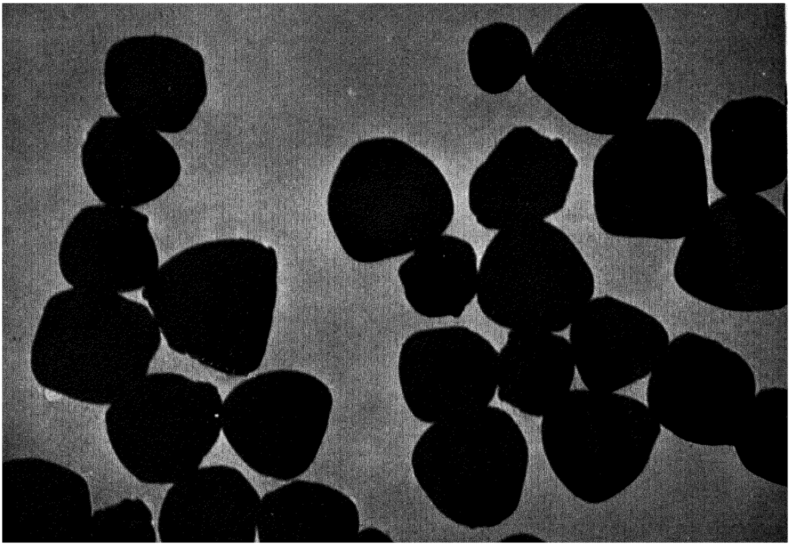

Fig. 1. Polyhedral bodies of *Choristoneura fumiferana* Clem.). × 10,000.

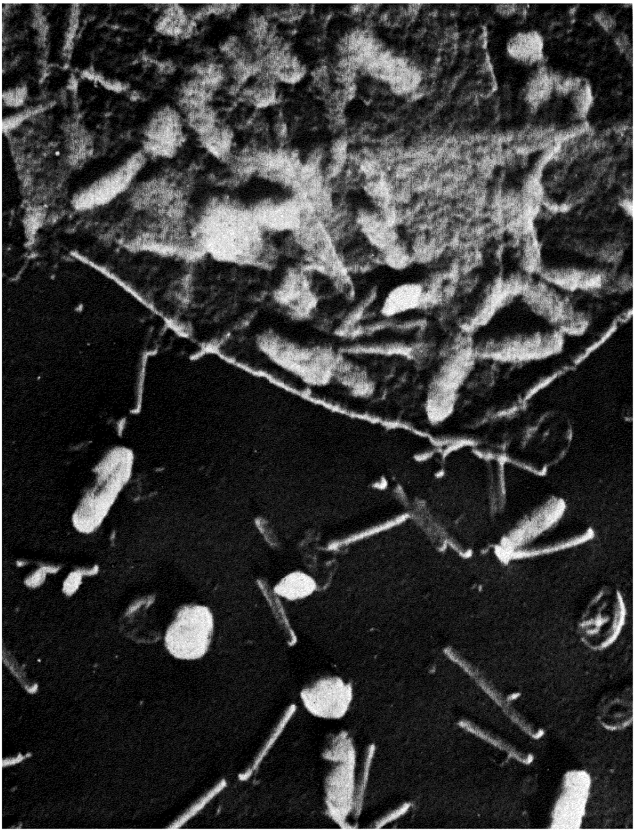

Fig. 2. Polyhedral bodies of *Porthetria dispar* (L.) in dissolution. Virus particles of various development stages inside and outside of the polyhedral body. × 33,000.

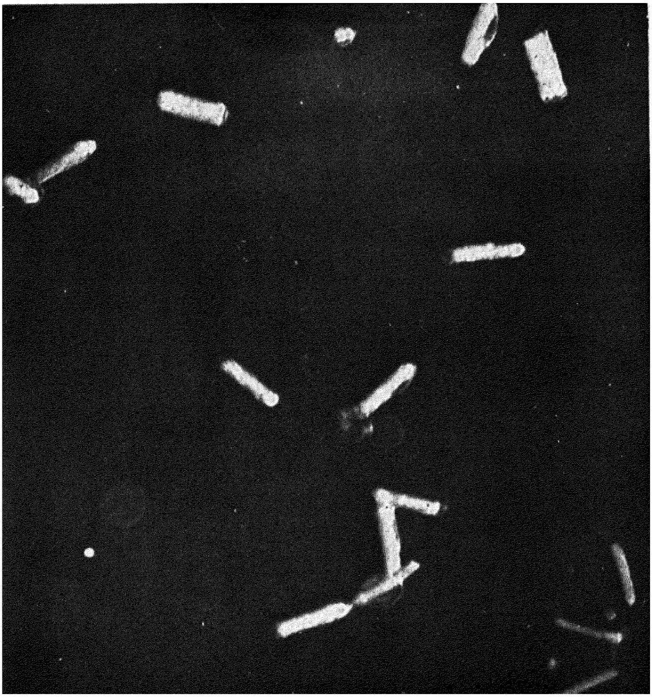

Fig. 3. Polyhedral virus particles of *Bombyx mori* (L.). Spherical developing stages, single and double rods within the developmental membrane, mature rods and empty membranes. × 33,000.

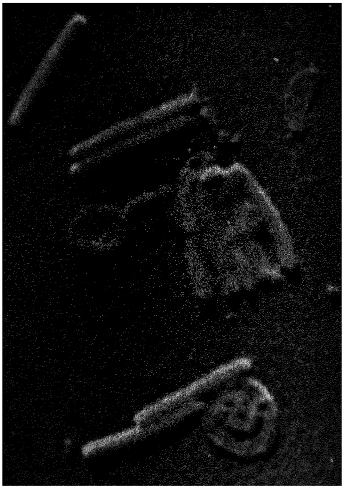

Fig. 4

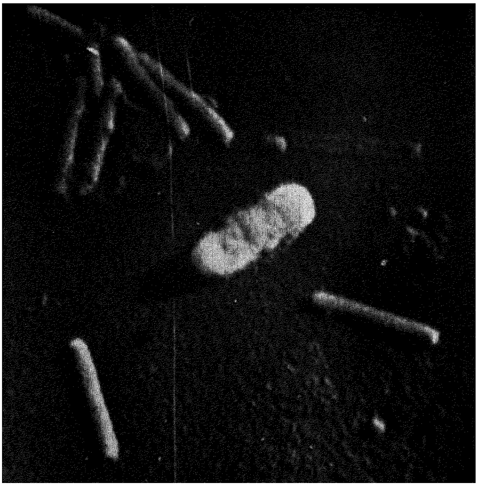

Fig. 5

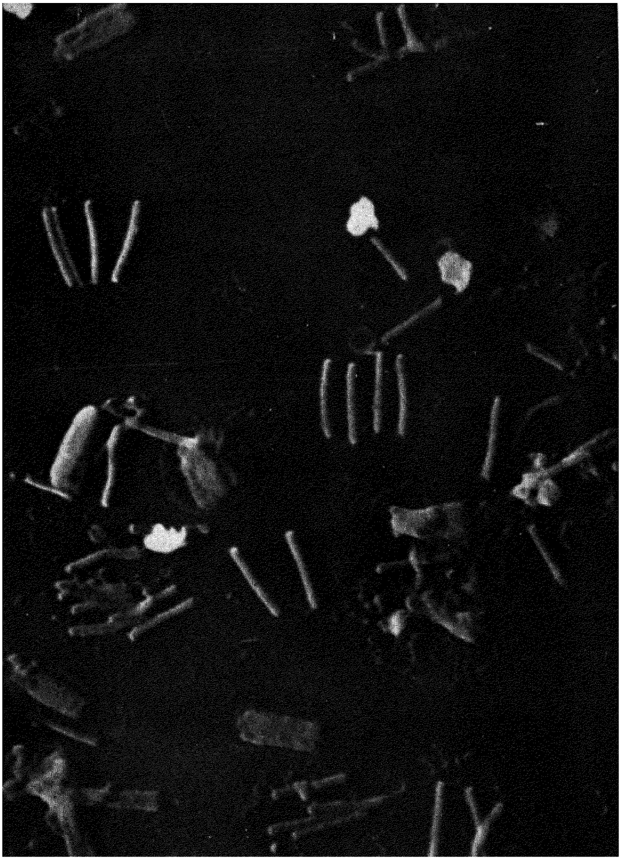

Fig. 6

Figs. 4–6. Polyhedral virus particles of *Porthetria dispar* (L.). Bundles with and without developmental membrane, some single virus particles disintegrate in spherical sub-units leaving the empty, intimate membrane. Figs. 4 and 5, × 50,000. Fig. 6, × 33,000.

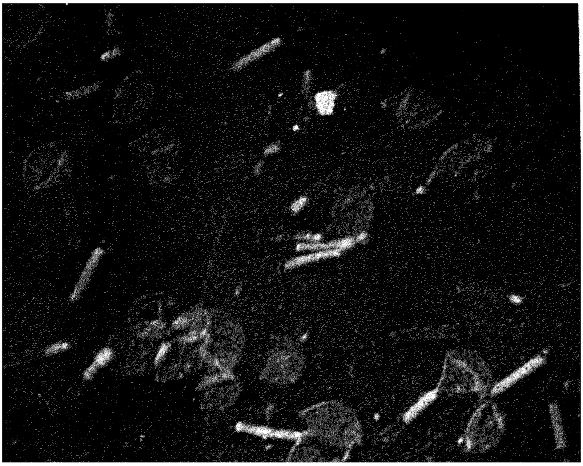

Fig. 7

Figs. 7, 8. Polyhedral virus particles of *Bombyx mori* (L.). Rods releasing the spherical sub-units, empty developmental and intimate membranes. × 33,000.

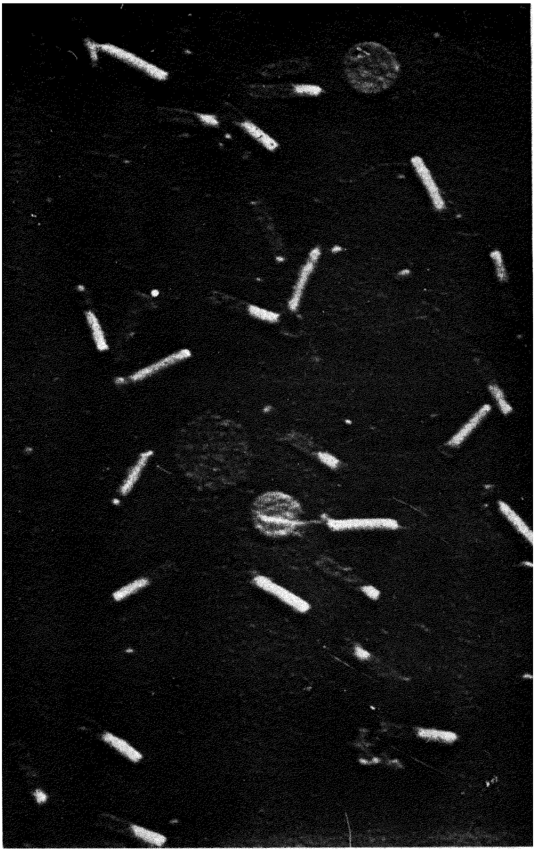

Fig. 8

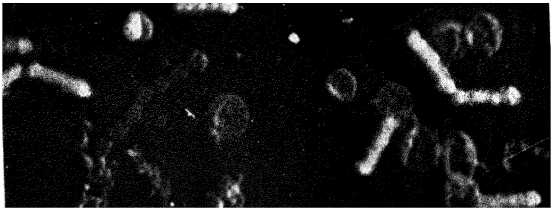

Fig. 9. Polyhedral virus of *Bombyx mori* (L.) showing spherical structure of the rods and intimate membrane after treatment with trypsine. × 33,000.

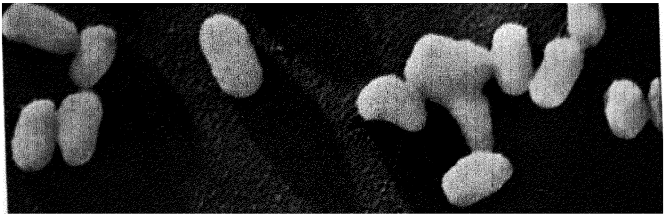

Fig. 10. Capsules (granules) of *Choristoneura fumiferana* (Clem.). × 25,000.

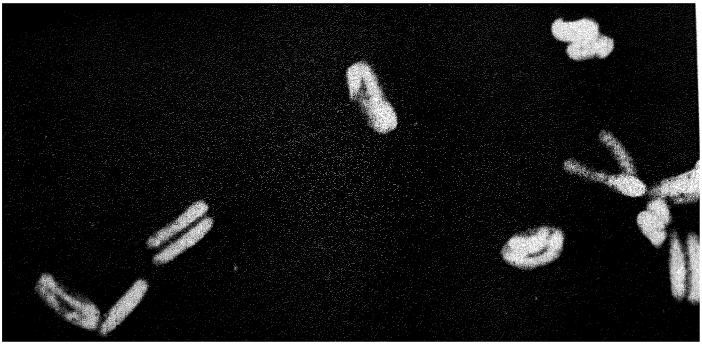

Fig. 11. Capsule virus of *Cacoecia murinana* (Hb.). Spherical developing stages, single and double rods within the developmental membrane. × 50,000.

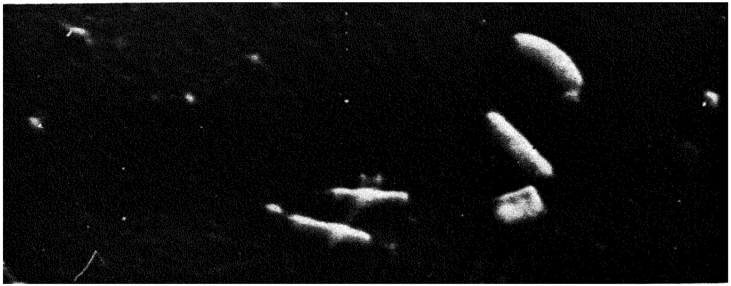

Fig. 12. Capsule virus particles of *Choristoneura fumiferana* (Clem.). Some single rods are disintegrating, releasing spherical sub-units. × 50,000.

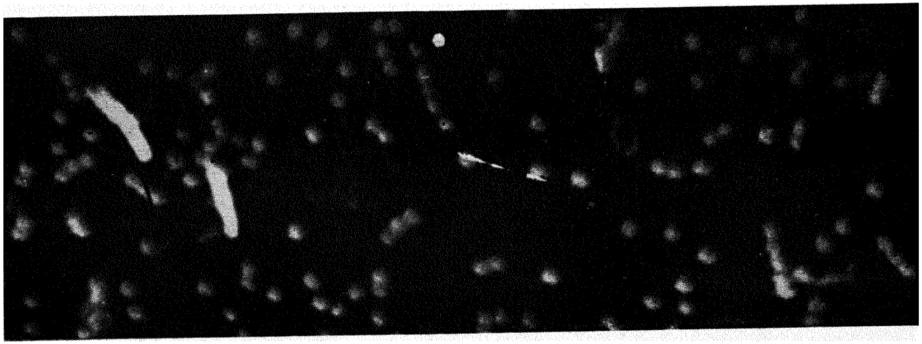

Fig. 13. Virus particles of *Arctia villaca*. Spherical particles, some rods, and empty developmental membranes. × 50,000.

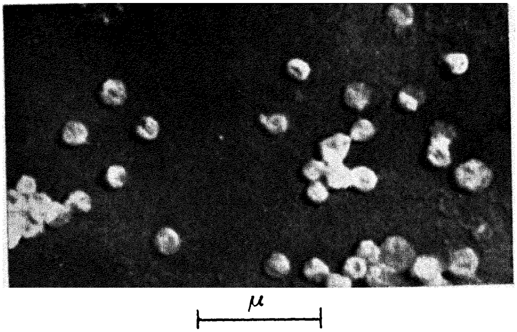

Fig. 14. Newcastle disease virus in water.

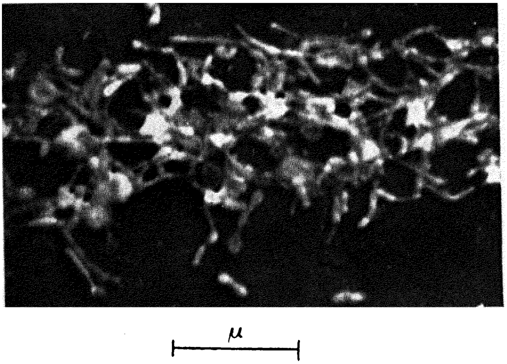

Fig. 15. Newcastle disease virus in 0·85 % NaCl.

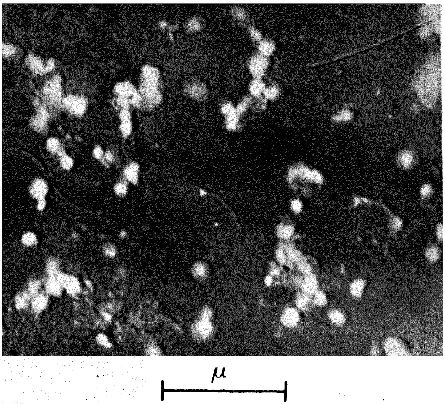

Fig. 16. Purified Newcastle disease viruses kept in distilled water for two weeks. Note that some of the viruses show a dense centre and a less dense periphery.

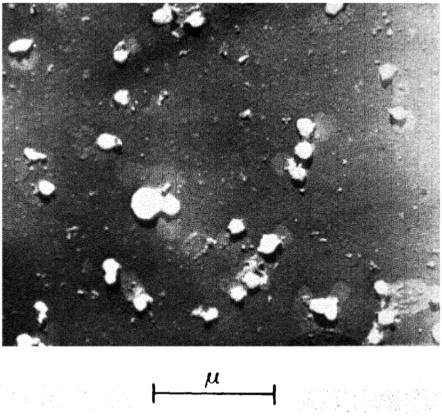

Fig. 17. The same virus preparation as in Fig. 16 after heating at 75° for 5 minutes. Note that some very thin structures seem to have been cast off from the virus particles.

Horsfall, F. L. (1949). Viral multiplication. *Federation Proc.*, **8,** 518.

Hoyle, L. (1948). The growth cycle of influenza virus A. A study of the relations between virus, soluble antigen and host cell in fertile eggs inoculated with influenza virus. *Brit. J. Exp. Path.*, **29,** 390.

Hoyle, L. (1950). The multiplication of influenza viruses in the fertile egg. *J. Hyg.*, **48,** 277.

Hughes, K. M. & Thompson, C. G. (1951). A granulosis of the omnivorus looper, *Sabulodes caberata* Guenee. *J. Infect. Dis.*, **89,** 173.

Luria, S. E. (1950). Bacteriophage: An essay on virus reproduction. *Science,* **111,** 507.

Markham, R., Matthews, R. E. F. & Smith, K. M. (1948). Specific crystalline protein and nucleoprotein from a plant virus having insect vectors. *Nature,* **162,** 88.

Putnam, F. W. & Kozloff, L. M. (1950). Biochemical studies of virus reproduction. IV. The fate of the infecting virus particle. *J. Biol. Chem.*, **182,** 243.

Rake, G. & Jones, H. (1942). Studies on lymphogranuloma venerum. I. Development of the agent in the yolk sac of chicken embryo. *J. Exp. Med.*, **75,** 323.

Smith, K. M. & Wyckoff, R. W. G. (1950). Structure within polyhedra associated with insect virus diseases. *Nature,* **166,** 861.

Smith, K. M. & Wyckoff, R. W. G. (1951). Electron microscopy of insect viruses. *Research,* **4,** 148.

Steinhaus, E. A. (1949). Nomenclature and classification of insect viruses. *Bact. Rev.*, **3,** 203.

Steinhaus, E. A., Hughes, K. M. & Wasser, H. B. (1949). Demonstration of the granulosis virus of the variegated cutworm. *J. Bact.*, **57,** 219.

Tang, F. F. & Wei, H. (1937). Morphological studies on vaccinia virus cultivated in the developing egg. *J. Path. Bact.*, **45,** 317.

Wasser, Harriette B. & Steinhaus, E. A. (1951). Isolation of a virus causing granulosis in the red-banded leaf roller. *Virginia J. Sci.*, **2,** 91.

Wyatt, G. R. (1950). *Studies on insect viruses and nucleic acids.* Thesis, University of Cambridge, England.

Wyckoff, R. W. G. (1951). Electron microscopy of chick embryo membrane infected with PR-8 influenza. *Nature,* **168,** 651.

DISCUSSION

A. FELIX: Is it possible to demonstrate the presence of polyhedral protein in the absence of virus? This would indicate which comes first.

G. H. BERGOLD: Yes, there are some claims (Smith & Wyckoff, *Research*, 1951, **4,** 148) that some polyhedra in certain insects seem not to contain any virus particles. However, these are exceptional cases and need confirmation.

T. E. MITTLER: With regard to the possibility suggested by Dr Bergold that the various particle sizes of insect viruses represent cyclical stages in the development of the viruses (rather than the simultaneous production of virus particles having a different expression of form) does any one form predominate in any one cell at a certain time after infection?

G. H. BERGOLD: This question is difficult to answer for two reasons: (1) The life and multiplication cycle of the virus—sphere, rod, spheres—takes place more than once in a single cell, (2) the shape of virus particles at intervals of time after infection is very difficult to investigate in an insect larva, since different organs and cells of one organ are not attacked simultaneously. One blood cell nucleus may show chiefly spherical forms but other blood cells at the same time are not affected or display spheres and rods. This problem can only be investigated in tissue cultures which are in progress in our laboratory.

N. W. PIRIE: The facets that appear on these polyhedral bodies resemble in many ways the facets that are found on starch grains in plants. Reichert (*Publ. Carnegie Instn.*, 1913 & 1919) found that each plant produced a characteristic shape of grain and that only a few produced the quasi-rhombic-dodecahedron which is the shape that would appear if plastic spheres were squashed together. He could not discover what controlled the precise shape and I wonder whether you find that the shape is characteristic of the virus and of the host and have any explanation of it.

G. H. BERGOLD: The shape of polyhedral bodies is rather characteristic of each host insect. This cannot be decided definitely since it is not certain whether insect viruses are transmitted to other than their specific hosts. Occasionally the shape of polyhedra is in a few nuclei quite different from that of the majority of the same individual insect larva.

Concerning the shape of the polyhedra, one might suggest that its crystal form may depend on the different shape and size of the polyhedral protein molecules (Bergold, *Z. Naturforsch.*, 1947, **2b**, 122).

H. P. CHU: Recently we have been interested in the effect of salt concentration on the morphology of the Newcastle disease virus first described by Bang (*J. Exp. Med.*, 1948, **38**, 291), who found that Newcastle disease viruses appeared spherical in water but became tailed and filamentous in form when suspended in saline. In recent years much interest has been directed to the filamentous form of animal viruses and its important implication on the mode of virus multiplication. It seems worthwhile to investigate more thoroughly how far the morphology of a virus is subjected to the change of the suspending fluid and why. We have therefore repeated Bang's work. Figs. 14 and 15 show how striking the Newcastle disease virus changes reversibly from the rod form in 0·85% NaCl solution to the spherical form in water. In order to see how much the osmotic pressure is responsible for the change we have examined the virus in 6% glucose solution which is isotonic to 0·85% NaCl. In this case also the viruses appear mainly as rod forms. The result shows that the osmotic pressure at least plays an important part in this morphological transformation. The additional fact that the change is reversible seems to suggest that there might be some limiting membrane in the Newcastle disease viruses.

It is interesting to note that mitochondria also change from rod form to spherical form when the salt concentration or osmotic pressure of the medium is reduced. It is generally believed that there is a limiting membrane in mitochondria which has a high content of phospholipids. One wonders whether Newcastle disease viruses and influenza viruses have a limiting membrane of the same nature. If so, would this membrane be reproduced in the same way as the membrane of the mitochondria, or is it simply a bit of the host cell membrane carried away by the virus particles when they leave the host cell as was suggested by Hoyle? What is the biological function of the membrane?

Some of our electron micrographs of purified Newcastle disease virus (Fig. 16) do show a distinct differentiation of a dense centre and a less dense, rather flat periphery. Elford *et al.* (*Brit. J. Exp. Path.*, 1948, **29,** 590) have also observed that some Newcastle disease virus has a slightly raised centre surrounded by a flat structure which they regarded, however, as mucinous substance rather than as a membranous structure. We have attempted to see if one can strip off this surface layer or membrane from the rest of the virus particle. Salton & Horne (*Biochim. Biophys. Acta,* 1951, **7,** 19) have recently succeeded in rupturing and separating the cell wall of bacteria by sudden heating. We have just treated a Newcastle disease virus preparation in the same way and obtained some rather encouraging results. Fig. 17 shows that some very thin structures seem to have been cast off from the virus particles after being heated at 75° for 5 minutes. If this preliminary finding can be substantiated by further observation, it would strongly indicate that there is a limiting membrane in the Newcastle disease virus.

K. ZINNEMANN: What happens to insect viruses during the early stages after injection into larvae? Does the virus disintegrate as other viruses seem to do or is it recoverable all the time?

G. H. BERGOLD: Efforts were made to demonstrate particles suspended in silkworm serum. Up to 3 hours both unchanged and disintegrating rods were found, but later the serum precipitates and then virus cannot be detected.

L. DMOCHOWSKI: Have you attempted the separation of smaller (spherical) particles from the larger (rod-shaped) particles and the estimation of their respective infectivity?

G. H. BERGOLD: The separation was tried by ultracentra-centrifugation, but without success so far.

THE MULTIPLICATION OF THE FOWL TUMOUR VIRUSES

J. G. CARR

British Empire Cancer Campaign Unit, Poultry Research Centre, Edinburgh

Most of the studies on virus multiplication have been made on the necrotizing viruses, where the cycle is simply infection—virus multiplication—cell death and virus release. But many viruses also stimulate cell proliferation—e.g. vaccinia—and Andrewes (1934) has called attention to the fact that a series can be drawn in both the epithelial and connective tissue infections which starts with purely necrotizing viruses, through those causing both necrosis and proliferation in various degrees, to others causing an almost pure malignant growth. In these last examples an exceptionally close relationship between the cell and virus multiplication cycles must hold: for if the virus multiplication rate were excessive the increasing accumulation of it should eventually overwhelm the cell and cause death, while if the cell were to outgrow the virus it would promptly be halted by lack of the stimulus to growth. This balance may not necessarily be a fine adjustment, for an alternating equilibrium of either type or both would serve to give the unrestrained proliferation of cells found in these infections.

This should not be regarded as implying that the cycle of virus multiplication is controlling or controlled by the nucleus of the cell directly, for an indirect link-up through the mitochondria, for example, could give the same result as far as our present knowledge goes.

There is even greater difference between the necrotic viruses and some of the proliferative ones such as the Shope papilloma virus and the avian sarcoma group, and that is that the latter will not infect a susceptible cell when they come into contact with it. Injury is necessary before infection takes place, and the resulting cycles of virus multiplication are necessarily entirely intracellular.

YIELD OF VIRUS FROM TUMOURS

The mass of tumour cells produced by the avian sarcoma viruses provides excellent material for studying the yield of virus per infected cell. When this was done (Carr, 1947) the best yield of virus reported was $3{\cdot}75 \times 10^7$ infective doses per gramme of tissue for the Rous 1 tumour (Claude & Rothen, 1942). Taking the volume of a tumour cell as $(10\mu)^3$

a cell content of 10^9 cells per cm^3 or per gramme of tumour tissue is obtained. This rough calculation shows that only about one cell in twenty can contain infective virus, and this was definitely a maximum yield, for most workers on this and the other avian sarcoma viruses report yields corresponding to 10^4–10^5 infective doses per gramme, i.e. one infective dose from several thousand cells. On the other hand, calculation from the amount of particulate material that was chemically, physically, and immunologically like the virus that was obtained corresponded to a dry weight of 4×10^{-13} g. per infection. The weight of the virus, calculated as a 70 mμ diameter sphere of density 1·3 should be $2{\cdot}3 \times 10^{-16}$ g. This corresponds to 1 infective particle to 574 similar but non-infective particles, or an average of about 28·7 such particles per cell. Rather better yields of virus since reported by Carr & Harris (1951*b*) still fall below one particle per cell, and only modify the calculation slightly.

It is now much easier to appreciate that this finding is in line with the general picture of virus multiplication. The proportion of 0·2% of the 'purified' virus that was actually infective was a strong discouragement to any investigation of the cause of the induced malignancy by chemical analysis of the virus. If the average content of all cells in the tumour is 28 particles, then it seems likely that this implies that cells about to divide into two will contain more than 28 particles and those which have just divided will contain less. Furthermore, if there is a soluble phase or disaggregated stage during the virus multiplication cycle, cells containing such would not contribute to the total of particles isolated, and therefore the particle content of the rest would have to be correspondingly higher. Even if no allowance is made for such a stage, the proportion of infective to non-infective particles is about 1:574, and this should also indicate the proportion of the life cycle of the virus, and of the cell mitotic cycle, in which fully-formed infective virus is present. This is such a short period of the cell mitotic cycle (1/574th of a 24-hr mitotic cycle is about 2½ minutes) that it is at once suspected that the stimulus to cell division is not only the fully-infective virus, but also some portion of the earlier stages of virus synthesis. It is not therefore possible to decide from this type of data how many particles are needed to cause a given cell to become malignant.

LOCATION OF VIRUS IN THE CELL

The fowl tumour viruses are below the resolution limit for ordinary optical microscopes, and inclusion bodies are not formed. Claude, Porter & Pickels (1947) detected bodies of the size of the virus in Tumours 1 and 10 by the use of the electron microscope. These particles were found in the cytoplasm of 48-hr tissue cultures, though because of thickness of the central region it could not be ascertained whether tl were also present in the nucleus. They did not state whether all c contained the virus, and the large number found is very much above average found by direct isolation, though not necessarily above the pectation if a prolonged soluble phase occurs. Immunological exp ments (see below) suggest that some nuclear material is incorpora in the virus, which perhaps points to a nuclear origin.

COMPOSITION OF THE VIRUS

It was shown by Gye & Purdy (1931) and confirmed by Amies (19 that antisera to normal fowl tissues would neutralize the tumour viru Using the Fujinami virus, which will grow in both fowls and ducks was possible to demonstrate that fowl-grown virus had a fowl cc ponent, while duck-grown virus had a duck component and not a f component (Gye & Purdy, 1933). Amies & Carr (1939) showed t the normal tissue component was present in the (nucleated) red c and not in the plasma (the statement by Haddow (1944) that the cc ponent is in the serum cannot be substantiated). When this was the o example known of this participation of the host tissue in the virus it used to produce a number of highly complex hypotheses about a 'd nature' or cytoplasmic origin of the virus. Since this participation host material has been found in other viruses, it can no longer be u for separating the avian sarcoma viruses into a separate class of entit or for supposing that the tumour viruses have a special mode of creati

MULTIPLICATION OF VIRUS AFTER INFECTION

In discussing the evidence available from past work, it has been assun that the multiplication of the sarcoma viruses in the cell follows the sa mechanism as other viruses. I have recently been able to secure evide that this is so (Carr, unpublished). The method used resembled that u for other viruses and may be briefly described. The host employed a newly-hatched chick, and an injection of about 1000 infective do was made into one leg. Larger doses were considered inadvisable, as host cell, probably the macrophage, might not otherwise appear in su cient number to deal with all the virus. At stated intervals, the chi were killed, and the leg muscles minced, extracted in 4 ml. distilled wa (which lyses all cells) in a small macerator, cleared by light centrifu tion, and titrated in more young chicks by the method described by C & Harris (1951*a*). It was found that recovery of virus added to extra of normal muscle, and the virus recovered a few hours after injecti into the leg was complete, within the accuracy of the assay. Despite the unhandy nature of the material, the transience of the infective stage, and the difficulties associated with the large number of chicks needed at once, a consistent story emerged from the later assays.

Table 1. *Infectivity of 0·2 cc. leg extract after hours:—*

Expt. No.	Initial Injection Infective Doses in 0·2 c.c. extract	0	6	12	18	24	36	42	48	60	66	72	84	108
1.	400	—	(2/2)	—		—	(1/2)	—	—	(3/3)	—	—	—	10^3(5/5)
2.	400	—	—	(2/2)		(1/2)	(2/2)	—	10(2/2)	(3/3)	—	—	10^2(1/2)	—
3.	400	10^2(1/3)	—	—		(3/6)	—	(3/6)	—	—	10(1/5)	10(5/5)	—	—
4.	40	10(2/3)	—	—		—	—	—	(2/3)	—	(2/3)	—	—	—

The figures in brackets indicate the number of animals injected (denominator) and number of tumours produced (numerator). The preceding figure (if any) indicates the dilution of the extract which produced these tumours.

— Indicates no virus detected in undiluted extract.

A summary of the results is given in Table 1. Within 6 hours after injection of virus into the leg the titre of the leg extract fell to about 1% of the amount of virus injected, and remained at this level for about 66 hours, when a sharp rise occurred. There are sufficient data in the literature to indicate that extractable virus is present thereafter. This fits in well with the histological findings (e.g. Levine, 1939) that the first detectable signs of malignancy (as opposed to the ordinary foreign body reaction) become recognizable at a period of about 55 hours or rather later. The subsequent histological data indicate a varied response of the cells, which discourages the hope of obtaining a 'second step' increase of virus. All pathologists seem to be agreed that subsequent increase is by cell multiplication, and not cell infection (which would anyhow need a renewal of the injury stimulus), and although there is a small amount of virus in the bird, this can easily be attributed to liberation as a result of the usual necrosis of tumour cells; in fact, the amount of virus in the whole of the normal tissues amounts to that in about 1 mg. of tumour tissue (Carr, 1944*a*).

FACTORS INFLUENCING VIRUS CONTENT OF TUMOURS

The yield of virus from a tumour therefore depends on the continued multiplication both of virus and cells. It seems that three main factors control the yield of virus from a tumour:

1. The serum antibodies which will neutralize the virus when extracted from the cells. These only become effective in highly-

immune birds, which in practice means only after 30 days growth of the tumour (Carr, 1943*a*).

2. The age of the host. Duran-Reynals (1946*a*) made the important observation that tumours in old birds produce little or no virus.
3. The rate of growth of the tumour, as slow tumours are likely to have fewer cells at the stage at which fully-formed and infective virus is present.

Evidence for the effect of the last two factors is provided by an analysis of the results of assay of thirty-seven Rous 1 tumours carried out between March and November 1951. All were induced by a large inoculum of cells and virus, so that growth-rate was mainly determined by the susceptibility of the host. All were assayed after 13–29 days of growth, i.e. at tumour ages where antibody has little effect. No relation between effect, activity and age was in fact found. Some of the experiments were from other lines of work, and some were from tumours chosen deliberately to investigate these effects.

The yield of virus is that given by a 10% extract, decimal dilutions of which were inoculated (0·2 ml.) into young chicks. The figures are rounded off to the nearest active decimal dilution. Both age of host and size of tumour were effective. In Table 2, the effect of age of host is shown, while the tumours are roughly classified in size.

Table 2. *Effect of age of host and tumour size on virus content of tumours*

Age of host (Days)	Activity of extract					
	Up to 10^3		10^4		10^5 and over	
	L	S	L	S	L	S
Up to 69	—	1	6	3	8	2
70–79	—	—	4	1	3	—
Over 80	4	2	1	—	2	—

The figures indicate the number of hosts falling in each group.
L, S = large and small tumours respectively.

The size of the tumour is, of course, only an approximate reflection of the growth-rate at the moment when the tumour was taken, and it has been necessary to ignore any variation due to the other factors of the host. It is clear that the tendency is for large and small tumours of old hosts to have a reduced virus content (except in very rapidly growing tumours) while conversely small tumours have a low virus content except in the younger birds. The experiments included eight pairs in which two birds of equal age received identical injections. In two pairs, the tumour size and virus yield were similar, while in the others the smaller tumour always contained less virus. One experiment involved two such pairs and summarizes all the findings.

Table 3. *Effect of age of host and tumour size on virus content of tumour*

Age of host (Days)	Tumour size	Virus yield of extract
87	Large	10^4
87	Small	10^1
34	Large	$> 10^5$
34	Small	10^5

As previously mentioned, all birds had received a large inoculum and the tumours were taken within a relatively narrow time range, so that the tumour size was an approximate reflection of the rate of growth of the tumour. That this really was the case was shown by other experiments where two inoculations of different amounts of virus (approximately 1:1,000) into the same bird gave both a large and a small tumour, but the relative growth-rates were similar (the size of tumours in the same host is proportional to the amount of virus injected). Extracts of the tumours gave the same yield of virus per gramme of tumour from both the large and small tumours in the same host. The size of the tumour is therefore not the critical factor, but its rate of growth.

It might be suspected from this that the actual virus content of a tumour is not identical in all parts, but that small pieces might have different contents depending upon local conditions in the tumour. It is interesting to note that Duran-Reynals,(1946*b*) has shown that mutations of the Rous virus are more frequent in older birds. A possibly similar aspect of the age effect is the finding of Gye (1931, 1932) that the Fujinami sarcoma grows readily in young ducklings but not in older birds. It is uncertain to what extent this is a general characteristic of viruses. Though they usually produce disease more often and more severely in young animals, some of this at any rate is probably due to the production of antibodies in adults. A summary of the relationship of age and infection is given by Duran-Reynals (1946*c*).

SUSCEPTIBILITY OF HOST

Once infection is established, the final outcome usually depends upon the genetic susceptibility of the host, antibodies having no effect upon intracellular virus. In the majority of cases some at least of any species will survive any infection. A small percentage of fowls will usually survive the Rous 1 sarcoma virus, and it has been shown (Greenwood, Carr & Blyth, 1948) that this is an inherited condition. Infection by the virus undoubtedly occurs, but the tumour fails to progress very far and usually regresses quite suddenly. It has been proved that the virus remains latent in such birds, which remain carriers with a high antibody content in their serum (Carr, 1943*b*). This resistance may break down, and development of normal virus-containing tumours occur years later. Such tumours recur at the site of inoculation, indicating that the carried virus is not disseminated among the tissues of the host (Carr, 1942*a*). The amount may be considerable, as laying carriers will pass large amounts of antibody through each egg (Carr, 1944*b*). There is as yet no information about the rate of virus multiplication in such birds.

NON-MALIGNANT CONDITIONS

The chick tumour viruses do not always produce malignant growth. Milford & Duran-Reynals (1943) have shown that in very young chicks and in embryos a necrosis of some cells of the blood vessels may result, seen as a haemorrhagic spot or large haematoma. They have succeeded in transmitting the virus as a purely necrosis-inducing form for several passages. The yield of virus from such lesions is small, but it is impossible to decide how many cells are involved, and even a small injury to a blood-vessel will produce a spectacular haematoma in the fowl. This finding seems to be in line with the previous suggestions, that virus multiplication is greater in the cells of young animals, and that excessive multiplication will result in necrosis of the host cell.

For many years, most poultry pathologists have been convinced often from purely clinical observations, that fowl paralysis or avian neurolymphomatosis is a manifestation related to the sarcoma-leukaemia group of avian viruses. Vasquez-Lopez (1936) suggested that involvement of the nervous system follows intracerebral inoculation of Rous 1 virus, and Duran-Reynals (1947) has produced a neurotropic variant of the Rous 1 virus. Greenwood & Carr (1951) consider that a mutation of the Rous virus can occur in fowls that are carriers of the virus and under certain conditions give rise to neurolymphomatosis in birds hatched from their eggs. In keeping with the low rate of reproduction of nerve cells, such neural infections always result in tissue destruction rather than proliferation, but the techniques of handling those neurotropic forms are not sufficiently advanced for any quantitative data on virus multiplication to be available.

ACTION OF CHEMICALS ON VIRUS MULTIPLICATION

Attempts to modify the yield of virus by treatment of the tumours with colchicine, cyanide, or by maintaining isolated pieces of tissue at 37° or 0° for many hours have not had any success (Carr, unpublished). Until a blockage leading to the mass production of infective virus can be evolved, investigations of the virus as isolated from tumours by chemical methods seem pointless. A more interesting effect is that produced by treating a 'carrier' bird with methylcholanthrene (Carr, 1942*b*). A violent local reaction occurs, leading to the production of a large typical Rous tumour in about 10 days. Grafts of such tumours contain infective virus. This stimulating effect of the carcinogen on the host cells of the virus and thus on the virus itself is perhaps similar to the action of oestrogens on the host cells of the mouse mammary virus.

It is interesting to speculate on the possibilities of producing 'incomplete' virus by such means. If infection were followed by the establishment of a carrier state, with the latent virus losing the potentialities of forming complete infective virus, then stimulation by a chemical carcinogen might set off multiplication of the host cell and non-infective form of the virus, giving a virus-induced tumour from which no *infective* virus can be isolated. This may have happened in the case mentioned previously of the old fowls of Duran-Reynals bearing non-infective tumours; perhaps also in the Shope papilloma where it has been shown that carcinogens will stimulate the malignant transformation in domestic rabbits (Rous & Friedewald, 1941). Such tumours contain something immunologically resembling the virus though infection cannot be demonstrated (Kidd, 1942).

Many years ago, Andrewes (1939) postulated 'toothless' viruses as one aspect of cancer causation, i.e. viruses which can cause proliferation but will not infect new cells. It is now easier to see that such viruses might arise from infective forms as a result of the mode of reproduction of viruses. Such a concept can be extended beyond the true cancer viruses, for Duran-Reynals (1950) has some indications that fowl-pox in carrier birds may result in an abnormal response to chemical carcinogens.

Such multiplication of 'incomplete' virus recalls the 'neoteny' of zoologists, where a larval form develops the ability to reproduce. But a virus reproducing without the property of infectivity is a difficult subject for experiment, especially if an outside stimulus is also involved. This is not a new problem to be faced, for in 1928 Boycott said 'we have no proof that the particles we get out of a lesion are the direct descendants of those we put in. In other words, we have to reopen the discussion question which most of us regarded as settled: is the agent the cause of the disease or the disease the cause of the agent?'

REFERENCES

ANDREWES, C. H. (1934). Viruses in relation to the aetiology of tumours. *Lancet*, ii, 63.

ANDREWES, C. H. (1939). Latent virus infections and their possible relevance to the cancer problem. *Proc. Roy. Soc. Med.*, **33**, 75.

AMIES, C. R. (1937). The particulate nature of avian sarcoma agents. *J. Path.*, **44**, 141.

AMIES, C. R., & CARR, J. G. (1939). Immunological experiments with highly concentrated suspensions of the Rous 1 tumour-producing agent. *J. Path.*, **49**, 497.

BOYCOTT, A. E. (1928). The transition from living to dead: the nature of filtrable viruses. *Proc. Roy. Soc. Med.*, **22**, 55.

CARR, J. G. (1942*a*). Observations upon spontaneously recurring Rous No. 1 tumours. *Brit. J. Exp. Path.*, **23**, 206.

CARR, J. G. (1942*b*). The effect of some substances influencing cell activity on the growth of the Rous No. 1 sarcoma. *Brit. J. Exp. Path.*, **23**, 221.

CARR, J. G. (1943*a*). The relation between age, structure, and agent content of Rous No. 1 sarcomas. *Brit. J. Exp. Path.*, **24**, 133.

CARR, J. G. (1943*b*). Prolonged antibody production following recovery of fowls from Rous No. 1 sarcoma. *Brit. J. Exp. Path.*, **24**, 138.

CARR, J. G. (1944*a*). The tumour virus disseminated from Rous No. 1 tumours. *Proc. Roy. Soc. Edin.*, B, **62**, 51.

CARR, J. G. (1944*b*). Lack of transmission of avian tumour virus from carrier hens to their offspring via the egg. *Proc. Roy. Soc. Edin.*, B, **62**, 54.

CARR, J. G. (1947). An unexplained discrepancy between the actual and expected yield of viruses from tumours and its implications. *Proc. Roy. Soc. Edin.*, B, **62**, 243.

CARR, J. G. & HARRIS, R. J. C. (1951*a*). The preparation and assay of the Rous No. 1 sarcoma agent. *Brit. J. Cancer*, **5**, 83.

CARR, J. G. & HARRIS, R. J. C. (1951*b*). Preservation of the agent of Rous No. 1 sarcoma by freeze-drying. *Brit. J. Cancer*, **5**, 95.

CLAUDE, A., PORTER, K. R. & PICKELS, E. (1947). Electron microscope study of chicken tumor cells. *Cancer Res.*, **7**, 421.

CLAUDE, A. & ROTHEN, A. (1942). Properties of the causative agent of a chicken tumor XIV. Relation between a tumor nucleoprotein and the active principle. *J. Exp. Med.*, **71**, 639.

DURAN-REYNALS, F. (1946*a*). Transplantability and presence of a virus in spontaneous fibromas and sarcomas of chickens in relation to the age of the tumor-bearing animal. *Cancer Res.*, **6**, 529.

DURAN-REYNALS, F. (1946*b*). The age factor in adaptability of a sarcoma virus to other animal species. *Science*, **103**, 748.

DURAN-REYNALS, F. (1946*c*). Age and infection—a review. *J. Gerontol.*, **1**, 358.

DURAN-REYNALS, F. (1947). A study of three new duck variants of the Rous chicken sarcoma. *Cancer Res.*, **7**, 99.

DURAN-REYNALS, F. (1950). V^{e} Congrès international du cancer, Paris.

GREENWOOD, A. W. & CARR, J. G. A possible connexion between the Rous No. 1 sarcoma and fowl paralysis. *8th World's Poultry Congress*, Paris.

GREENWOOD, A. W., CARR, J. G. & BLYTH, J. S. S. (1948). Indications of the heritable nature of non-susceptibility to Rous sarcoma in fowls. *Brit. J. Cancer*, **2**, 135.

GYE, W. E. (1931). A note on the propagation of Fujinami's myxo-sarcoma in ducks. *Brit. J. Exp. Path.*, **12,** 93.

GYE, W. E. (1932). The propagation of Fujinami's myxo-sarcoma in ducklings. *Brit. J. Exp. Path.*, **13,** 458.

GYE, W. E. & PURDY, W. J. (1931). *The cause of cancer.* London: Cassell & Co.

GYE, W. E. & PURDY, W. J. (1933). Infective agent in tumour filtrates: a further investigation by means of antisera to normal tissues. *Brit. J. Exp. Path.*, **14,** 250.

HADDOW, A. (1944). Transformation of cells and viruses. *Nature*, **154,** 194.

KIDD, J. G. (1942). The detection of a 'masked' virus (Shope papilloma virus) by means of immunization. *J. Exp. Med.*, **74,** 321.

LEVINE, M. (1939). The cytology of the tumor cells in the Rous chicken sarcoma. *Amer. J. Cancer*, **36,** 236.

MILFORD, J. J. & DURAN-REYNALS, F. (1943). Growth of a chicken sarcoma virus in the chick embryo in the absence of neoplasia. *Cancer Res.*, **3,** 578.

ROUS, P. & FRIEDEWALD, W. F. (1941). The carcinogenic effect of methylcholanthrene and of tar on rabbit papillomas due to a virus. *Science*, **94,** 495.

VASQUEZ-LOPEZ, E. (1936). On the growth of Rous sarcoma inoculated into the brain. *Amer. J. Cancer*, **26,** 29.

DISCUSSION

L. DMOCHOWSKI: In connection with Dr Carr's remarks on the multiplication of fowl tumour viruses as well as with comments made by other speakers, it may be of interest to mention some of our observations on the mammary tumour inducing agent of mice or the Bittner virus.

The first observation relates to the development of spontaneous breast tumours in mice of a subline of C57 black low-breast-cancer strain after six years of observation during which no breast tumours were recorded in any of the four hundred breeding females of this strain. As already reported (Dmochowski, *Acta Union Intern. contre le Cancer*, 1951, 7, 230), the first two C57 black strain female mice which developed breast tumours, as well as the subsequent tumorous females, were found to be descendants of the same original C57 black female, whose ancestry was free from cancer. The first two breast tumours when tested for the presence of the Bittner virus in suitable test mice failed to reveal the presence of the virus. It was then concluded that the genetic and hormonal factors may have been responsible for the appearance of breast tumours in these low-cancer-strain mice.

Shortly after the development of the first two breast tumours, more tumours were recorded, gradually bringing the total of spontaneous tumours in this C57 black subline to twelve. For purposes of demonstration a simplified table is shown (see Fig. 1) in which all the tumours are demonstrated. As can be seen from this table, not only the first two tumours, already mentioned, but also the two subsequent tumours (one in F3 generation, and one in F4 generation) failed to reveal the presence of the virus, when tested in similar susceptible test mice which give an incidence of 80–90% of tumours when injected with material containing the virus. Had only these four tumours been observed, the conclusion reached after the test of the first two tumours would have been strengthened and perhaps made final. Contrary to expectation, however, out of seven subsequent breast tumours in the F6 generation five revealed the presence of the virus. Again the tests of these seven tumours were carried out in test mice similar to those used in the tests of the first four breast tumours. In each test, the age of the test mice when injected with extracts of the breast tumours, the method of preparation of the tumour extracts, and the route of injection of the extracts were the same. Furthermore, the majority of the test mice in each negative test lived well above the age at which tumours develop. The age of C57 black strain mice at which the spontaneous breast tumours developed, the number of the litter to which the tumourous mice belonged, and the histological appearance of the tumours could not have influenced the results of the tests, as they were approximately the same and the tumours on histological examination revealed a similar structure. It can be stated therefore that the tests which failed to reveal the agent in the first four tumours were as adequate as the tests of the subsequent tumours, the majority of which were found to harbour the agent. An interesting point should be stressed: as can be seen from the table, sometimes three or even as many as seven tumour-free generations may intervene between two females which develop spontaneous breast tumours, although the tumour-free females (marked X on the figure) of the intervening generations have lived to an age at which tumours could have developed.

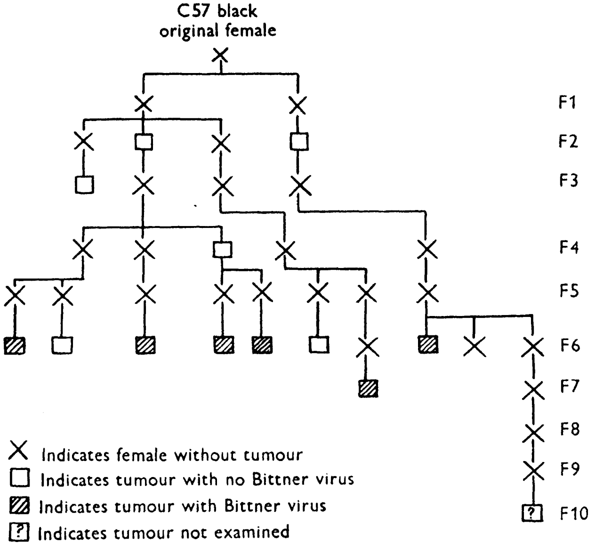
C57 black
original female
F1
F2
F3
F4
F5
F6
F7
F8
F9
F10
X Indicates female without tumour
☐ Indicates tumour with no Bittner virus
▨ Indicates tumour with Bittner virus
[?] Indicates tumour not examined

Fig. 1

For an interpretation of this observation on the 'appearance' of the Bittner virus in a low-cancer strain the following may be considered.

The virus could have been present constantly in a latent or 'masked' state or as symbiont in females of this strain or it could have been present in small amounts only and appeared under the influence of genetic or hormonal factors or both, or the resistance of the mice has broken down and the tumours have developed as observed by Dr Carr in fowls carrying the latent Rous agent. It could also be argued in the same way, as Dr Bawden and Dr Pirie do in discussing the origin of viruses that the Bittner virus has arisen endogenously by some accidental change in a normal cell component that prevented it from filling its normal role, but not from stimulating the synthesizing machinery of the cell, and here again the genetic and hormonal as well as other factors may have been responsible for the change. It is difficult to decide whether the agent was present in females of the C57 black low-breast-cancer strain as a symbiont or in small amounts, because female mice belonging to the ancestry of the original female developed no breast tumours after being subjected to intense hormonal stimulation by the so-called forced breeding, and further because few breast tumours appeared in female mice of this strain which had been treated with a carcinogenic hydrocarbon methylcholanthrene (Dmochowski & Orr, *Brit. J. Cancer*, 1949, **3**, 520), and in these carcinogen-induced breast tumours no virus was detected by means of infectivity tests similar to those already mentioned. In carrier birds treatment with methylcholanthrene induces Rous tumours which contain infective virus (Dr Carr). Assuming, however, that the virus was present in the first four breast tumours to be tested, the failure to extract the virus from these tumours may have been due to a close integration with the tumour cells so that it was unable to survive the death of the cell during extraction procedures. It has been suggested by Dr Bauer that failure to detect a virus in a neoplasm by infectivity tests need not necessarily mean its absence from the tumour, as the virus may be present in a 'soluble phase' without ever needing to assume a particulate form. This interpretation however encounters great obstacles in the difficulty of deciding what constitutes this so-called soluble phase, as even in the influenza virus the soluble phase appears to be particulate (Hoyle).

Our observation on the ' disappearance' of the virus from a transplantable breast tumour may also be mentioned. This tumour was originally induced in a C57 black low-breast-cancer strain female by foster nursing by an RIII high-breast-cancer strain female. The agent was found still to be present in the cells after 42 serial passages of this tumour in C57 mice lacking the agent (Dmochowski: *Brit. J. Cancer*, 1949, **3**, 246). In an attempt to ascertain whether the presence of the virus is a permanent feature of the transplanted cells, the transplantable tumour was tested again for the presence of the virus after 82 serial transplants. This test failed to reveal the presence of the virus in the tumour cells. It should be stressed that the histology of this tumour has remained unaltered. A similar observation on the disappearance of the rabbit papilloma virus from carcinomata which originated from papilloma cells has been reported by Smith, Kidd & Rous (*4th Intern. Cancer Res. Cong.*, 1947, p. 84). Serological tests in mice, similar to those in rabbits, in order to ascertain the presence of a latent or masked virus in cells of the 82nd transplant could not be carried out because of the observation that the sera of mice do not have antibodies against the agent, no matter whether the mice belong to high- or low-breast-cancer strains, whether they bear a tumour with the virus or whether they have been injected with material containing the virus (Dmochowski & Passey: *New York Acad. Sci.*, 1951, in the press). This is contrary to observations on fowls which have a high antibody content when the virus is latent in these birds (Carr). Here again several interpretations of the observed phenomenon could be put forward.

The virus may have changed somehow, possibly by mutation. It has become closely associated, if not fused, with one of the particulate elements of the tumour cell. Close integration of the virus with the tumour cells may have produced a state in which it was unable to survive the death of the tumour cells during extraction procedures preparatory to the test. The virus has indeed disappeared after serial passages through low-cancer strain mice, although it is difficult to understand why. The virus may have become 'toothless' as postulated by Dr Andrewes (*Lancet*, 1934, ii, 63), that is, a virus which may cause proliferation but will not infect new cells. In agreement with Dr Carr it may be stated that a virus reproducing without the property of infectivity may be a difficult subject for experiment.

In conclusion it can be stated that the observed appearance and disappearance of the Bittner virus may possibly be connected with the mode of multiplication of other tumour viruses. It is difficult at the present stage of our knowledge or ignorance of the multiplication of tumour viruses to say how it is connected. Any of the interpretations which have been mentioned, although stimulating, need not necessarily be, or lead to, a true explanation.

R. LATARJET: Dr Carr's experiments and those of Duran-Reynals and of Rous & Friedewald which he quoted, disclosing a rapid promoting action of methylcholanthrene on virus-carrying animals, recall some similar facts which have been observed with other carcinogenic agents. Such are these strange experiments of Lacassagne & Vinzent (*C. R. Soc. Biol.*, 1929, **100**, 249) confirmed by Burrows, *et al.* (*Proc. Roy. Soc.* B., 1937, **123**, 213), which showed that a single mild X-irradiation (600 r) produces in rabbits a high rate of malignant tumours at the site of the irradiation when the tissues have been previously prepared with some non-specific inflammatory agent. I observed that mice which had been prepared six months before by repeated localized ultraviolet irradiations, responded to very few, and even to a single painting with methylcholanthrene, by developing, within one to three weeks, rapidly growing *sarcoma* on the treated area.

Now, coming back to the case of virus-induced tumours, Dr Carr suggests that the chemical might set off multiplication of cells which would contain the virus in a non-infective form. As carcinogens usually behave on cells as potent growth inhibitors rather than growth promotors, I wonder whether such effects are not due to an action on the growth of the virus—to some kind of unmasking process, for instance, —rather than on the growth of the host.

POSSIBLE MULTIPLICATION CYCLES IN NEUROTROPIC VIRUSES

F. K. SANDERS

Department of Zoology and Comparative Anatomy,
University of Oxford.

INTRODUCTION

When infectivity is the property used to measure virus sensitivity, the assault of a virus on a susceptible cell can often be described in terms of four successive phases: (*a*) a phase of 'adsorption' and penetration, during which virus particles in the extracellular, maximally infective, phase become irreversibly attached to the surface of cells and somehow penetrate within them; (*b*) a 'constant' phase, during which little or no virus infectivity can be recovered from cells when they are broken and extracted in various ways; (*c*) a period of rapidly increasing infectivity, the 'rising' phase; followed by, (*d*) a phase during which the new crop of infective particles is released from the cells.

A definite succession of phases will usually only be detected when we can follow events occurring simultaneously in a large number of similar cells which have all been infected at the same time. Thus, most of our information concerning virus growth cycles comes at the present time from the study of bacterial viruses, which can be propagated in suspensions of isolated cells (Delbrück, 1947; Luria, 1950), and of influenza virus, which grows readily in the relatively simple system provided by the chorio-allantoic membrane of the chick embryo (Hoyle, 1948, 1950; Henle & Henle, 1949). Such studies have given rise to the hypothesis that virus replication takes place mainly in the constant phase, and that, in the case of the bacterial viruses, it is preceded by the breakdown of the invading virus particles into smaller units which in some way then organize production by the cell of a pool of replicas of themselves which, with further constituents, will eventually be combined to make up the increased number of new infective particles which is released at the end of the cycle. Such a working hypothesis has been proposed for bacterial viruses by Luria (1947), and subsequently adopted by Burnet (1951). For influenza it has also been suggested that there is a precursor of the completed virus particle in the form of a non-infective, 'soluble' phase, possibly identical with the 500 S sedimentable antigenic component (Hoyle, 1950). The details of the multiplication of these viruses will no doubt be dealt with by other members of this Symposium, and I mention them here only for the reason that if such facts can be established for viruses as remote from one another in the scale as bacterial viruses and influenza, it becomes important to enquire whether replicative processes of this somewhat unusual kind are characteristic of the family of viruses as a whole. If it can be shown that this is so, not only will a character have been found at last by which to distinguish viruses formally from other classes of organisms, but we may have to think again concerning the plausibility of the Green-Laidlaw hypothesis of the origin of viruses by degeneration of parasitic organisms of a higher order of complexity.

This paper is concerned with attempts which have been made to find evidence of a growth cycle of the above type in several neurotropic viruses. We have been especially interested in a group of small viruses which, although unrelated antigenically, have all roughly the same small size (diameter $= c.\ 10\ \mathrm{m}\mu$), and grow readily in the cells of the central nervous system of various species. These viruses include the two types of agent (TO; GD VII) causing the disease of mouse encephalomyelitis (Theiler, 1937; Theiler & Gard, 1940), the viruses of the encephalomyocarditis group (Dick, 1949; Warren, Smadel & Russ, 1949), and poliomyelitis virus. Most of our experiments have been done with the GD VII strain of mouse encephalomyelitis virus, and with encephalomyocarditis virus, hereinafter called EMC. Neither virus grows well in the chorio-allantoic membrane of the chick embryo, and we have had to use a living mammalian host, the mouse. In contrast to experiments performed on isolated systems, those conducted *in vivo* offer particular hazards. In most cases the only source of virus available is a crude suspension of infected tissue which contains much apart from active virus particles. The tissue injected contains many kinds of cell differently situated as regards susceptibility and availability for infection: the number of susceptible cells reached by a given inoculum is never accurately known; and, finally, the whole result may be complicated by nutritional and genetic factors, or by an immune response on the part of the host organism. These difficulties should warn us to use extreme caution in interpreting the results of experiments such as those I shall describe. Nevertheless, it appears probable that neurotropic viruses may have multiplication cycles with features similar to those shown by bacterial viruses or influenza. At the present, however, it is only possible to demonstrate the bare framework of the cycle. Later experiments with more refined techniques—particularly with suitable tissue cultures—may reveal whether the details of the multiplication of these viruses can also be described in similar terms.

MULTIPLICATION OF MOUSE ENCEPHALOMYELITIS VIRUS

Most of the work to be described has been done with the GD VII strain of mouse encephalomyelitis virus (Theiler & Gard, 1940). This virus is strongly neuronotropic, and has a particular affinity for the anterior horn cells of the spinal cord. It is able to migrate along neuronal pathways (Sanders, 1952). After injection into a muscle, virus travels to the central nervous system along the fibres of the nerve supplying that muscle, eventually arriving at the cells of origin of the nerve, in which the first outburst of virus multiplication in the nervous system presumably takes place. Evidence summarized elsewhere (Sanders, 1952) indicates that virus possibly travels within the axons themselves. After intramuscular injection virus penetrates nerve fibres at the periphery, possibly at the neuromuscular junctions.

The property of neuronal transport, which this virus shares with poliomyelitis (Howe & Bodian, 1941) seems less mysterious when we consider it in relation to the unique structure of the adult nerve cell. The axon, which is merely an extension of its cytoplasm, may be anything up to a metre long. We may thus liken the nerve cell to an Amoeba, one of whose pseudopodia is drawn out to a length of anything up to 10^4 cell diameters. If we accept the proposition that virus travels within the axons, then the penetration of virus from an injected muscle into its nerve, its migration to the central nervous system, and its first cycle of multiplication therein, can be regarded as analogous to what happens when influenza virus becomes attached to, penetrates within, and multiplies inside, a number of cells of the chick embryo chorio-allantoic membrane. The difference is that in the case of the neuronotropic virus, attachment of virus to the cell surface takes place at a place remote from that at which virus multiplication ultimately occurs. Viruses with this property may thus exemplify a condition where, owing to anatomical factors, the ‘adsorptive’ phase of the growth cycle is prolonged. The irreversible union of the virus particles with the cell surface and the passage of virus into the cell, is succeeded by a phase in which the virus travels to the specific situation within the cell where conditions are appropriate for its multiplication. GD VII virus presents us with an opportunity for studying virus in this phase, *i.e.* between the point at which it penetrates the cell, and that at which it undergoes multiplication.

Many factors, including age of the host organism, influence the penetration of virus from an injected muscle into the axons of its nerve (Sanders & Macdonald, 1952). They are as yet little understood, and it is not proposed to discuss them further at present. It might be profitable, however, to consider from this point of view the various constitutional barriers to invasion of the central nervous system by neurotropic viruses described by Sabin (1941) and his collaborators.

Once inside the nerve GD VII virus travels to the nervous system at about 0·2 mm. per hour (Sanders, 1952). A study has been made of the distribution of infective virus along the course of the sciatic nerve at intervals after injection of a standard dose of virus into the gastrocnemius muscle. The evidence obtained indicates that no loss of infectivity accompanies the migration phase. A wave of infectivity can be shown to move up the nerve at a rate comparable with the rate of virus travel estimated by other methods. In these experiments groups of ten mice were killed at intervals after injection and the sciatic nerve from the muscle entry up to the sciatic notch removed from each mouse. The nerve was then cut up into ten segments, the segments from comparable levels from each of the ten mice pooled, and their virus content estimated by standard methods. The virus content at each level in the nerve could then be expressed in LD_{50} per mg. of tissue. In this way histograms could be prepared relating the virus content of different segments of the nerve to the level from

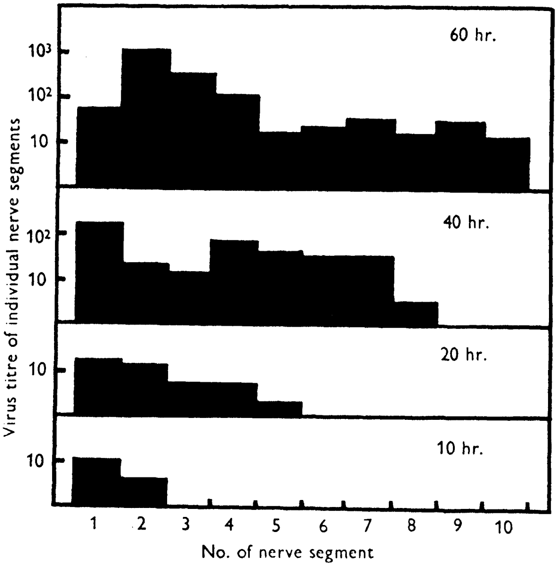

Fig. 1. GD VII virus. Virus content at different levels in the sciatic nerve at intervals after injection of a standard dose of virus into the gastrocnemius muscle. Muscle to the left in each case.

which the segments were taken at different intervals after injection. Fig. 1 shows the distribution of infective virus along the sciatic nerve, 10, 20, 40, and 60 hours after injection of this large dose of virus into the right gastrocnemius. At 10 hours, virus was not found in any but the two lowest segments of the nerve. Consideration of Fig. 1 shows that after injection there is a rise in the concentration of virus at the lower end of the nerve. This reaches a maximum at about 60 hours after injection and then declines. This may be a result of the multiplication of virus peripherally in the muscle as claimed by Rustigian and Pappenheimer (1949) or to the transfer of more and more of the original virus injected into the lower end of the nerve; the original inoculum contained about 3,000,000 intracerebral LD_{50} per mg. However, long before a maximum concentration is reached peripherally, virus can be recovered from all levels in the nerve, although in low concentration. This indicates that virus has reached the central nervous system long before the peripheral changes have come to an end, and that this virus is still infective. This contrasts with what happens when the virus reaches the perikaryon of the cell itself (see below).

Interruption of the nerve fibres rapidly abolished their ability to transport virus. Two groups of mice were given the same intramuscular dose of virus. Immediately afterwards the right sciatic nerve was crushed as close to the spinal cord as possible in all the mice of one group. Forty-eight hours later all the mice were killed, and the virus content at different levels of the nerve determined in the two groups. In the control group virus was present throughout the nerve, although the upper third of the nerve contained very little. Only the lower third of the crushed nerves contained any measurable infectivity. It was thus clear that crushing directly after inoculation prevented the virus from travelling more than one third of the way up the nerve; moreover that found in the lower part may not be intraneural migrating virus, but virus which has leaked into the perineural spaces from the site of injection (see Sanders, 1952). Since 48 hours had passed between nerve interruption and necropsy, it was possible that loss of fibre continuity as a result of Wallerian degeneration was responsible for the effect observed. Histological examination showed that at this time the peripheral stump of the divided nerve contained many myelin 'ovoids' and 'unduloids' (Young, 1944), but there were many fibres which appeared intact over long distances. We have at the moment no direct evidence that in mice any fibre retains complete continuity from the muscle to the level of crushing at this time, but in mammals generally the power to conduct impulses does not begin to disappear until about 50 hours after nerve severance, and is usually not complete by 90 hours after section (Heinbecker, Bishop & O'Leary, 1932). So it is possible that after nerve crushing the ability of nerve fibres to transport virus disappears before the ability to conduct impulses, and long before the histological signs of degeneration appear.

A study has also been made of the fate of the migrating virus upon its arrival within the central nervous system, using the technique of intralingual inoculation. Most of the motor innervation of the mouse tongue comes from a compact group of nerve cells lying far dorsally in the medulla oblongata, and the region containing them can be easily identified by external landmarks. After intralingual injection virus travels via the hypoglossal nerve, and an outburst of virus multiplication takes place in these cells. At intervals after injection in this way, a group of five mice was killed and their brains removed aseptically. The brain was then sliced down to a small fragment of tissue which contained little apart from the hypoglossal nuclei already mentioned, and the dorsal vagal nuclei which overlie them. The fragment of tissue so obtained from one mouse weighed about 4 mg. The tissue fragments obtained from five mice killed at the same time were then pooled and the quantity of infective virus which they contained estimated by standard methods.

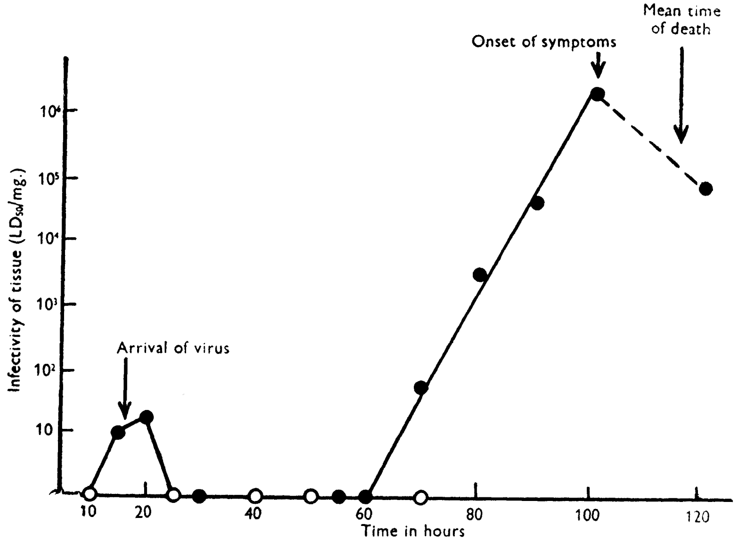

Fig. 2. GD VII virus. Titration of virus infectivity in the region of the hypoglossal nucleus at intervals after injection of virus into the tongue. Open circles:—no virus detectable in sample tested. Filled circles:—virus present. Filled circles on the base line represent amounts of less than 1 LD_{50} per mg.

By plotting the virus titres so obtained against the time elapsing between tongue inoculation and the time at which each group of mice was killed, it was possible to follow the changes in virus concentration which occurred in this part of the nervous system. The result of a typical experiment of this kind is shown in Fig. 2. From the known rate of migration of GD VII virus and the length of the nerve concerned it could be calculated that virus should arrive in this part of the nervous system after about 30 hours. It will be seen that after 20 hours about 10 LD_{50} of virus infectivity per mg. of tissue was, in fact, recoverable from this site, and it is assumed that this represents the small fraction of the original inoculum which had succeeded in penetrating into the axons of the hypoglossal nerve and had been transported within them to the central nervous system. The appearance of active virus in the nucleus was, however, immediately followed by a rapid decline in infectivity, so that between 30 and 60 hours after tongue injection either no virus, or else only a very small quantity (1 LD_{50} per mg. or less) could be demonstrated. From 60 hours onwards, however, a rapid local increase in infectivity occurred so that at 100 hours over 1,000,000 LD_{50} per mg. was present. The attainment of a maximal virus concentration coincided closely with the first appearance of symptoms (tongue paralysis).

The phase of minimal infectivity of about 20 hours which followed the arrival of virus may be analogous to the 'constant' period in the multiplication cycles of bacterial virus or of influenza, 'virus' being present in the hypoglossal nuclei throughout the period of low infectivity, although altered in some way so that its infectivity is no longer demonstrable. The length of the constant period, however, seems to be much greater than that of bacterial viruses (less than one hour) or of influenza (two to six hours). Such a difference, however, need not reflect any fundamental disparity in the method of reproduction employed by the two viruses, but may simply be a result of widely different metabolic conditions within the infected cells. For example, the respiratory rate of mouse brain (expressed as $mm^3.O_2$/mg. dry-weight/hour), is 11,000 whereas that of *Bact. coli* is approximately twenty-five times as great (271,000), (Krebs, 1934). The constant period of GD VII virus on the basis of our figures is about 30 hours, forty to one hundred and fifty times longer than the constant period of bacterial virus. The constant periods of the two viruses thus differ by a factor of the same order as the respiratory rates of the cells in which they grow.

An alternative hypothesis to account for the phase of minimal activity might, however, be that virus passes on through the hypoglossal nuclei leaving only minimal traces of its passage, and then multiplies elsewhere within the nervous system, returning to this region only at a later date.

An attempt has been made to decide between these two hypotheses in the following way.

Mice were inoculated intralingually as before and at 48 hours (i.e. in the middle of the period of low infectivity), were divided into two groups, killed, and the standard tissue fragment removed. In one group its cells were frozen before dissection while in the other dissection was carried out as far as possible without injury to the tissue. The pieces of tissue obtained were then transplanted individually to the brains of a second set of mice which were observed for twenty-one days and those showing specific symptoms, recorded. Histological examination of the brains for lesions caused by GD VII virus was then carried out. The result of one such experiment is shown in the accompanying table. Five out of six mice, which had received transplants of unfrozen hypoglossal nuclei initially, succumbed with symptoms of encephalomyelitis, and all their brains showed histological signs of infection. Three out of four mice whose transplants had been frozen before transplantation survived without symptoms for the whole observed period; the fourth mouse died four days after transplantation, but its brain showed no lessions, death being clearly not due to virus.

Table. *GD VII virus. Demonstration of the presence within cells, during the latent period, of non-infective virus.*

Condition of transplanted tissue	Number of recipient	Day of death	Specific lesions
Not frozen	1	3	++
	2	3	++
	3	4	++
	4	4	++
	5	7	+
	6	*S*	–
Frozen before transplantation	1	4	–
	2	*S*	–
	3	*S*	–
	4	*S*	–

S = mouse survived for whole period of observation (21 days).

In further experiments, hypoglossal nuclei, removed from mice killed 48 hours after injection of GD VII virus into the tongue, were explanted into rocker flask tissue cultures and incubated for 48 hours. At the end of this time, pieces of tissue were removed from the cultures, frozen, homogenized, and the infectivity of the resulting suspension determined. A suspension made from cultures prepared from unfrozen nuclei contained 3,000 LD_{50} of virus infectivity per mg. of tissue harvested. Cultures made with hypoglossal nuclei which had previously been frozen, however, contained no demonstrable infectivity. Since the infectivity of a suspension of active virus is not affected by freezing, the results of both the above experiments indicate that the formation of infective virus in the isolated fragment of tissue depends in some way upon the integrity of the cells which it contains. Thus, during the period of minimal infectivity 'virus' must be present within the region studied, although its infectivity cannot be demonstrated.

The attainment of a high titre of virus in the nucleus coincides closely with the onset of tongue paralysis observed clinically, and with the appearance of histological lesions; the latter include loss of cytoplasmic basiphilia by the affected cells and shrinkage of their nuclei, the whole process culminating in cell lysis. However, during the constant phase and the period of rising infectivity which succeeds it, very little histological change can be seen, except, possibly, for an increase in the basiphilia of the cytoplasm in the ten hours immediately preceding the first appearance of histological lesions. This may indicate a change analogous to that described for bacterial viruses by Luria & Human (1950), who noted the appearance of granular accumulations of Feulgen-positive material in infected bacteria at the same time as the outburst of desoxyribosenucleic acid synthesis in the latter half of the constant phase which precedes and parallels the appearance of active virus particles.

An attempt has also been made to follow the cycle of development of GD VII virus in the hypoglossal nucleus by titration of the specific haemagglutinative activity of the virus particles (Lahelle & Horsfall, 1949). It seemed of interest to discover whether the growth cycle of GD VII virus included a precocious development of haemagglutinative capacity, as has been claimed for influenza virus (Hoyle, 1948; Henle & Henle, 1949). The result of one such experiment is shown in Fig. 3. The dotted line in this figure represents the curve of infectivity development already described, drawn to the same scale. It will be seen that the onset of development of haemagglutinative activity roughly coincides with the development of infectivity. Further experiments will be necessary before one can be certain whether there is a definite separation in the time of the return of these two properties of the virus particle.

We may summarize the infectivity cycle of GD VII virus as follows:—Penetration into the axons of susceptible cells is followed by transport of virus to the central nervous system. The invading virus particles do not lose their infectivity during this process, although the whole transport mechanism appears sensitive to loss of continuity between the axon and its cell body. Upon arrival in the cell body the virus presumably finds a

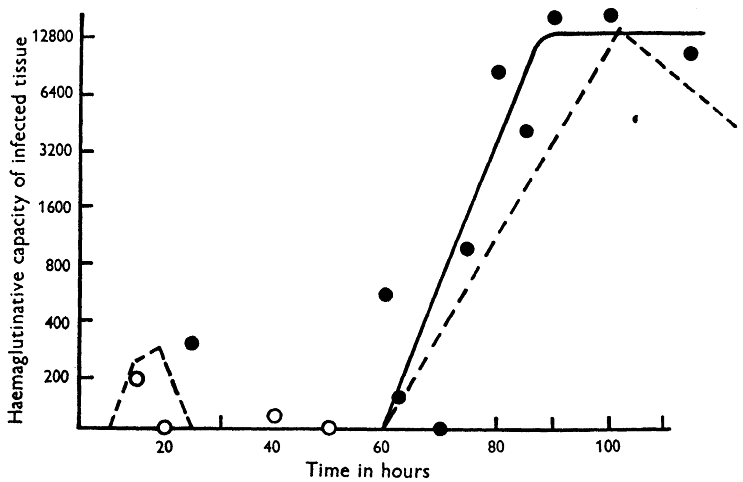

Fig. 3. GD VII virus. Titration of haemagglutin in region of hypoglossal nucleus at intervals after injection of virus into the tongue. Open circles:—no haemagglutination at dilution tested. Filled circles:—50% haemagglutination end-point.

favourable environment for its multiplication and infectivity is lost. There follows a constant period of some thirty hours after which there is a rapid increase of infectivity followed by cell destruction and the release of active virus.

ANALOGOUS CYCLES IN OTHER NEUROTROPIC VIRUSES

Poliomyelitis: When one turns to other neurotropic viruses it is difficult to find evidence of a growth cycle of this type. However a latent period may exist during the multiplication of poliomyelitis virus (Bodian & Cumberland, 1947). Although they do not comment on the fact, their figures show that in monkeys injected intracerebrally very little virus could be recovered from the central nervous system until the day before the onset of paralysis, when the virus content rapidly rose to a maximum. As in the case of GD VII virus, no specific changes in the infected cells could be found until this time (Bodian, 1949). Then, as with GD VII, the first change observed was the appearance of central chromatolysis in the nerve cell cytoplasm, resulting in the paling and eventual disappearance of the basiphilic Nissl bodies. Thereafter the cell might undergo sudden lysis or else be removed by phagocytes. Alternatively, it might recover, the cytological changes during recovery being very similar to those seen in the recovery from chromatolysis following axon section. During the chromatolysis caused by virus invasion, changes in the nucleus also occurred, including shrinkage and margination of chromatin. In cells destined for destruction the nucleus was eventually reduced to a small shrunken knot of basiphil material. In recovering cells a small basiphil body is often found—a type B intranuclear inclusion of Cowdry (1934)—whose nature is at present unknown.

EMC virus: An attempt has also been made to follow the development of EMC virus infectivity in the hypoglossal nucleus of the mouse following the injection of virus into the tongue (Sanders & Strahan, 1952). We are here faced with a somewhat different problem from that of GD VII virus since, so far as is known, EMC virus is not transported along nerve fibres to the central nervous system after peripheral injection but multiplies first in some peripheral locus—probably striated muscle—after which large quantities of virus are set free in the blood and reach the central nervous system by this route. Thus, in the case of EMC virus, the dissected tissue fragment containing the hypoglossal nuclei does not constitute the region of the central nervous system where virus first arrives from the periphery, but is merely a sample population of susceptible cells which probably become infected via the blood at the same time as the rest of the nervous system.

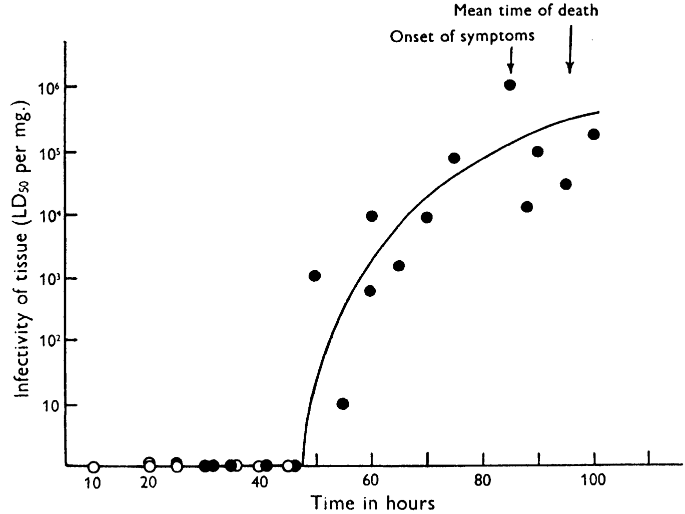

Fig. 4. EMC virus. Titration of virus infectivity in the region of the hypoglossal nucleus at intervals after injection of virus into the tongue. Symbols as in Fig. 2.

Fig. 4 shows the amount of virus infectivity recoverable from the hypoglossal nuclei at intervals after the injection of approximately 10^4 LD_{50} of virus into the tongue. For the first 20 hours no virus was recoverable;

from 25 to 45 hours only minimal quantities could be found (less than 1 LD_{50} of virus per mg. of tissue harvested); after 45 hours there was an extremely rapid increase in the quantity of virus present so that by 70 hours more than 10^4 LD_{50} per mg. were present.

Studies of virus distribution after the intramuscular injection of small quantities of EMC virus have shown that virus does not appear in the blood until 20 to 24 hours after inoculation. The precocious appearance of virus in the hypoglossal nucleus at 25 hours may thus represent virus in the blood-vessels of this part of the nervous system. Assuming that the cells of this part of the central nervous system become infected immediately virus appears in the blood-stream, we can date the earliest time of the infection of the hypoglossal nerve-cells as 25 hours after tongue inoculation, that is approximately 20 hours before there is a sudden increase of infectivity in this region. This would estimate the constant period of EMC virus to be approximately 20 hours. The constant small amount of activity recoverable during this period may be due to the blood-content of the tissue. This estimate of latent period accords well with what is known concerning the minimum incubation time of this virus, which, after the intracerebral injection of massive doses of virus, can be as short as 18 hours. Injection of very large amounts of virus into the central nervous system may thus result in the infection of so many cells that a single simultaneous cycle of virus multiplication takes place at the end of which the cells are destroyed and the mice show symptoms.

Herpes virus: Although herpes virus is also able to migrate along nerve fibres, no detailed study of the multiplication of herpes virus in a localized part of the central nervous system has yet been made. Burnet & Lush (1939), however, estimated the virus content of whole mouse brains at intervals after they had been injected with a known dose of the virulent HF strain of herpes virus. From their data a very approximate picture of the multiplication cycle can be obtained. Such a cycle is shown in Fig. 5. Infectivity disappears temporarily from the infected tissue but reappears 12–24 hours later and thereafter increases steadily for three or four days when death takes place. While this probably does not represent a single one-step cycle of virus multiplication, it is possible that the interval of about 12 hours,which elapses between the disappearance of infectivity and the first sign of new virus activity may represent the constant period of the first cycle.

The main interest of herpes virus from our present point of view is that it induces the formation of typical inclusion bodies in the nuclei of infected cells, and the correlation of the histological details of their development with the growth cycle of the virus may give clues concerning the mode by which the virus is replicated. I am indebted to the work of Goodpasture (1925), Crouse, Coriell, Blank & Scott (1950), and Scott *et al.* (1950) for the account of inclusion development which follows.

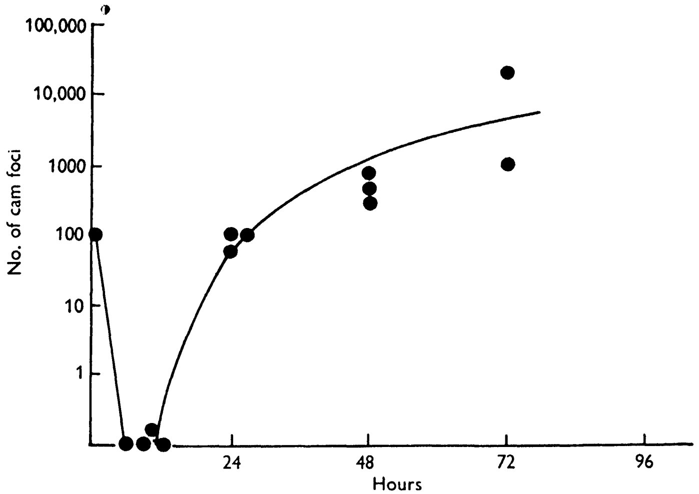

Fig. 5. Herpes virus (HF strain). Titration of virus infectivity at intervals after the injection of virus into the mouse brain. Titrations by pock-counting on the chorio-allantoic membrane (CAM) of the chick embryo. Data from Burnet and Lush (1939).

The earliest signs of change may be the appearance of a few minute granules within the nucleus, which stain red with eosin (Goodpasture, 1925). Later these grow larger, and fuse together, eventually occupying almost all the nucleus and becoming basiphil in their staining reaction; in this stage they give a positive Feulgen reaction. Still later, the inclusions lose their affinity for basic dyes, and revert once more to eosinophilia. It is only in this last stage that the affected cells show the type of inclusion considered 'typical' for herpes virus—a large acidiphil body occupying most of the nucleus of the infected cell, but separated by a clear halo from the nuclear membrane; the latter carries a dense deposit of basiphil material. The time relationships of this process are as follows:—

Before 10 hours no inclusions are seen. From then up to 16 hours a few cells may be found to contain acidiphil bodies, but between 16 and 24 hours there is a rapid increase in the number of cells with inclusions, the majority of these, moreover, are basiphilic. Later on there is a steady increase in the proportion of cells which have inclusions of eosinophilic type.

These changes may be tentatively related to the growth curve seen in mouse brain. This is, of course, a somewhat risky procedure since most of the cytological changes observed have been described in a variety of tissues other than brain. However, herpes virus seems to induce similar cytological changes in whatever susceptible tissue it is propagated and there is thus some little justification for this procedure. Nevertheless, it must be emphasized that the interpretation offered is purely an interim one, and must inevitably be modified when directly comparable histological and quantitative data become available. With these reservations, the first appearance of inclusions may be seen to occur at a point about two-thirds of the way through the constant phase. The period of rapid increase in the number of inclusions and the change to basiphilia would then coincide with the phase of rising infectivity.

Other cytological phenomena observed are an increase in the size of the nucleolus immediately preceding the appearance of inclusions and a positive response on the part of early inclusions to the Gomori test for alkaline phosphatase (Crouse *et al.*, 1950). Both of the latter appearances have been regarded by many authors (Caspersson, 1947) as indicative of intense protein synthesis on the part of the cells which show them. While the above cytological changes do not indicate absolutely that herpes virus multiplies within cell nuclei, the results of the experiments of Francis & Kurtz (1950) suggest that herpes virus may be associated not only with nuclei but with some part of the nucleoplasm other than the chromosomes, which is supported by the cytological evidence. Both cytological and quantitative observations would be consistent with the following interpretation:

1. That virus duplication does not occur until certain properties of the infecting particle, notably infectivity, have been lost;
2. That the appearance of desoxyribosenucleic acid is correlated with the resumption of infectivity; and
3. That the classical eosinophilic inclusion represents some product that is left behind after the new virus particles have been released from the cells.

FURTHER PRODUCTS OF CELL-VIRUS INTERACTION

Many virus and rickettsial infections cause the formation in infected tissues of serologically specific substances which are quite distinct from the infectious agents themselves. Such substances are normally termed soluble antigens, and have been found in at least five rickettsial diseases, and in infections caused by vaccinia, the viruses of the psittacosis-lymphogranuloma group, influenza, and lymphocytic choriomeningitis. It has been suggested that such substances may represent detachable fragments of the infectious agents themselves, and Hoyle (1950) identifies the soluble complement-fixing antigen of influenza with a precursor or incomplete form of the virus itself, since the antigen makes its first appearance in the growth cycle during the constant phase when no infectivity can, as yet, be demonstrated. However, the precocious appearance of the complement-fixing antigen has been denied by later authors. Moreover, immunization of animals with either of the two soluble antigens of the vaccinia virus neither induces protection nor elicits the production of neutralizing substances against the virus (Smadel & Hoagland, 1942). Similarly, the soluble antigen of lymphocytic choriomeningitis appears to play no part in resistance to this infection (Smadel & Wall, 1940). If soluble antigens represent incomplete forms of the virus particles it might be expected that their antibodies would exert some protective effect as has been suggested by Melnick (1951). There is, however, an alternative view of the nature of these soluble substances. If, as current opinion would suggest, virus reproduction takes place by the diversion of the protein synthetic mechanism of the cell by the virus or some constituent of it, one might regard soluble antigens as unusual by-products arising during the process of virus synthesis by the enzyme mechanisms of the cell, rather than as portions of the viruses themselves. It would therefore be of interest to discover whether the production of unusual non-infectious substances is in any way a general consequence of cell-virus interaction.

In the diseases so far mentioned the soluble antigens differ widely in chemical and physical properties, but are all smaller than the agents with which they are associated. However, as one goes down the list of viruses with respect to size it becomes increasingly difficult to find evidence of a 'soluble' substance separable from the virus itself by current techniques, and it is possible that with the smallest viruses the corresponding substances have either the same or even a greater particle size than the infectious agents themselves, or else are in some way bound to the tissue fragments removed from virus suspensions during the preliminary clarification of crude tissue extracts. Of particular interest in this respect are the virus-inhibitor substances found by Gard (1944) in crude extracts of the brains of mice infected with the virus of mouse encephalomyelitis and a similar inhibitor found in mouse brains infected with encephalomyocarditis virus (Sanders, 1950). Both viruses are extremely small and both inhibitors sediment with tissue fragments when brain segments are centrifuged at low speed. At the present time, however, it is impossible to be certain of the exact nature of such inhibitors. Recent experiments reported briefly by Melnick (1951) indicate that low speed sediments from the spinal cords of mice infected with the Lansing strain of poliomyelitis virus possess a binding capacity for poliomyelitis virus. Infected spinal cords were homogenized in 0·14 M sodium chloride and then centrifuged at low speed. A large amount of active virus was found in the supernatant. The sediment was then resuspended and subjected to as many as nine successive washings in this same saline; the virus content of the later washings became extremely low. At this stage extraction of the sediment with 1·0 M sodium chloride resulted in the appearance of a further outburst of virus activity in the supernatant, amounting to 10% to 50% of the activity present in the original extract. Control experiments involving the addition of the sediment from normal mouse spinal cords to a saline suspension of virus showed that the comparable fraction from normal tissue did not bind poliomyelitis virus. Apart from this there is very little evidence at present as to the histological localization of the accessory substances formed during virus multiplication. It would be interesting to know what relation (if any) they have to the non-virus intranuclear inclusions found in poliomyelitis and in mouse encephalomyelitis and the eosinophil inclusions found in the later stages of herpes infection (see above).

Mention should also be made of the remarkable polyhedral crystalline inclusions found in certain virus diseases of insects. Electron microscope examination, both of isolated polyhedra and of sections of infected tissue (Bergold, 1947, 1948; Smith & Wyckoff, 1950), has shown them to be complex structures in which little rods or spheres, consisting presumably of virus, lie embedded in a matrix of accessory substance. Further investigation of the chemical nature and serological properties of this substance might reveal an analogy with the soluble antigens.

Finally there is evidence that the replicative process itself may not always result in the production of an absolutely uniform set of particles. Non-neurotropic influenza virus undergoes only a single cycle of growth in the mouse brain, and there is also produced an incomplete product having haemagglutinative and complement-fixing activity, but no infectivity (Schlesinger, 1950; Cairns, 1951). Similarly, when influenza virus is passed serially on the chick chorio-allantoic membrane using massive inocula, mutual interference results in the production of a mixed virus population, some particles of which are fully infective, while others show only the other two properties (von Magnus, 1946). The extreme example of this process may be the pro-virus of lysogenic bacteria, whose infectivity is only sporadically acquired.

These facts serve to emphasize the concept that virus multiplication within the infected cell may give rise to a number of products, only one of which is infective virus capable of entering other cells and initiating further cycles of replication.

CONCLUSION

Study of the growth of neurotropic viruses inclines to the view that their multiplication is of the same general type as that postulated for other viruses. When a virus particle has succeeded in penetrating a susceptible cell, it travels to that part of the cell where it can best multiply itself. In the case of herpes virus this is probably the nucleus, in the cases of GD VII and poliomyelitis viruses, the cytoplasm of the nerve-cell. On reaching this site the virus particle is altered in some way so that it is no longer infective, and evidence available from a study of vaccinia virus (Crawford & Sanders, 1952) suggests that this change may have to do with the loss of nucleic acid from the virus particle. The resumption of infectivity also seems to depend on the addition of nucleic acid to the completed particles. We have as yet no evidence of the breakdown of neurotropic viruses into individual 'genetic' units as a preliminary to multiplication. Indeed, the existence of such phenomena as linkage in the genetics of bacterial viruses (Hershey & Rotman, 1948) suggests that perhaps the use of the term 'genetic' for the ultimate virus units need not be interpreted too literally. The analogue of a virus unit is not a gene, but an assemblage of genes; a better genetical equivalent for the replicative unit of a virus is perhaps a chromosome, or part of a chromosome.

If virus infectivity is correlated with the presence of nucleic acid on the virus particle, there is in fact quite a striking parallel between virus and chromosome behaviour. Viruses carry nucleic acid when in the extracellular infective phase; before replication they may lose it. Chromosomes carry a heavy nucleic acid charge during mitosis; in the interphase nucleus, when the cell is carrying on its normal metabolism and the genes are presumably exercising their functions, the amount of nucleic acid on the chromosomes is at a minimum. Whatever the truth or otherwise of this suggestion, it is quite clear that many of the analogies which have been made between various genetical entities and viruses in the extracellular infective stage (Darlington & Mather, 1949), are entirely misleading; the parts of viruses responsible for initiating replication are probably smaller than the infectious units, but may still contain many genes. Moreover we know practically nothing of the size and physical and chemical properties of viruses in the state when they are actually multiplying.

A somewhat neglected aspect of the growth cycle is the study of what happens when a cell recovers from a virus infection. Bodian (1949) for example, shows that in the case of two strains of poliomyelitis virus that multiply to the same titre in the monkey spinal cord, one, the Lansing strain, causes the utter destruction of a great many neurones; in the other case—the Frederick strain—a very high proportion of the infected neurones recover. In the early stages of infection by both strains, practically all the anterior horn cells show signs of virus invasion. We have virtually no idea what change in a cell enables it to resist invasion by one strain while it is destroyed by another, although both multiply in it to the same extent. In cases where sufficient cells escape destruction to enable the host to survive, there is usually a rapid disappearance of virus from the infected region once the last cycle of multiplication has run its course. In other cases, virus may persist in association with its host cell for very long periods. For example, the TO strain of mouse encephalomyelitis can be recovered from the infected central nervous system for as long as one year after infection (Theiler & Gard, 1940). Parallel with this prolonged association between host cells and potentially infective virus, we have to consider the case of incomplete virus. Lysogenic bacteria pass on prophage to their descendants even after serial culture in a medium containing an antiserum against the virus, yet such lysogenic cultures, appropriately stimulated by external agencies, can undergo lysis and release active virus (Lwoff, Siminovitch & Kjeldgaard, 1950).

REFERENCES

Bergold, G. (1947). Die isolierung des Polyeder-virus und die Natur der Polyeder. *Z. Naturforsch.*, **2b**, 122.

Bergold, G. (1948). Bundelformige Ordnung von Polyederviren. *Z. Naturforsch.*, **3b**, 25.

Bodian, D. (1949). The virus, the nerve-cell, and paralysis. A study of experimental poliomyelitis in the spinal cord. *Bull. Johns Hopkins Hosp.*, **83**, 1.

Bodian, D. & Cumberland, M. C. (1947). The rise and decline of poliomyelitis virus levels in infected nervous tissue. *Amer. J. Hyg.*, **45**, 226.

Burnet, F. M. (1951). Some biological implications of studies on influenza viruses. *Bull. Johns Hopkins Hosp.*, **88**, 119.

Burnet, F. M. & Lush, D. (1939). Studies on experimental herpes infection in mice, using the chorio-allantoic technique. *J. Path. Bact.*, **49**, 241.

Cairns, H. J. F. (1951). The growth of influenza viruses and Newcastle disease virus in mouse brain. *Brit. J. Exp. Path.*, **32**, 110.

Caspersson, T. (1947). The relations between nucleic acids and protein synthesis. *Symp. Soc. Exp. Biol.*, **1**, 127.

Cowdry, E. E. (1934). The problem of intranuclear inclusions in virus diseases. *Arch. Path.*, **18**, 527.

Crawford, G. N. C. & Sanders, F. K. (1952). The multiplication of vaccinia virus in tissue cultures of adult rabbit skin. *Quart. J. Micr. Sci.* (in press).

Crouse, H. V., Coriell, L. L., Blank, H. & Scott, T. F. M. (1950). Cytochemical studies of the intranuclear inclusion of herpes simplex. *J. Immunol.*, **65**, 119.

Darlington, C. D. & Mather, K. (1949). *The Elements of Genetics.* 1st edition. London. Allen and Unwin.

DELBRÜCK, M. (1947). Bacterial viruses or bacteriophages. *Biol. Rev.*, **21,** 30.

DICK, G. W. A. (1949). The relationship of Mengo encephalomyelitis, Encephalomyocarditis, Columbia SK, and MM viruses. *J. Immunol.*, **62,** 375.

FRANCIS, T. & KURTZ, H. B. (1950). The relation of herpes virus to the cell nucleus. *Yale J. Biol. and Med.*, **22,** 579.

GARD, S. (1944). Tissue immunity in mouse poliomyelitis. *Acta. med. Scand.*, **119,** 27.

GOODPASTURE, E. W. (1925). Intranuclear inclusions in experimental herpectic lesions of rabbits. *Amer. J. Path.*, **1,** 1.

HEINBECKER, P., BISHOP, G. H. & O'LEARY, J. L. (1932). Nerve degeneration in poliomyelitis. III. Rate of depression and disappearance of components of conducted action potential in severed nerves; correlation with histologic degeneration in groups of fibres responsible for various components. *Arch. Neurol. Psychiat.*, **27,** 1421.

HENLE, W. & HENLE, G. (1949). Studies on host-virus interactions in the chick embryo-influenza virus system. III. Development of infectivity, haemagglutination, and complement fixation activities during the first infectious cycle. *J. Exp. Med.*, **90,** 23.

HERSHEY, A. D. & ROTMAN, R. (1948). Linkage among genes controlling inhibition of lysis in a bacterial virus. *Proc. Nat. Acad. Sci.*, **34,** 89.

HOWE, H. A. & BODIAN, D. (1941). *Neural mechanisms in poliomyelitis.* 1st edition. New York. Commonwealth Fund.

HOYLE, L. (1948). The growth cycle of influenza virus A. A study of the relations between virus, soluble antigen, and host cell in fertile eggs inoculated with influenza virus. *Brit. J. Exp. Path.*, **29,** 390.

HOYLE, L. (1950). The multiplication of influenza virus in the fertile egg. *J. Hyg.*, **98,** 277.

KREBS, H. A. (1934). Atmung und Garung in lebenden Zellen. *Tabulae Biologicae*, **9,** 209.

LAHELLE, O. & HORSFALL, F. L. (1949). Haemagglutination with the GD VII strain of mouse encephalomyelitis virus. *Proc. Soc. Exp. Biol. Med., N.Y.*, **71,** 713.

LURIA, S. E. (1947). Reactivation of irradiated bacteriophage by transfer of self-reproducing units. *Proc. Nat. Acad. Sci.*, **33,** 253.

LURIA, S. E. (1950). Bacteriophage. An essay on virus reproduction in *Viruses, 1950* 1st edition, ed. M. Delbrück. California Institute of Technology, Pasadena.

LURIA, S. E. & HUMAN, M. L. (1950). Chromatin staining of bacteria during bacteriophage infection. *J. Bact.*, **59,** 551.

LWOFF, A., SIMINOVITCH, L. & KJELDGAARD, N. (1950). Induction de la production de bactériophages chez une bactérie lysogène. *Ann. Inst. Pasteur*, **79,** 815.

VON MAGNUS, P. (1946). Studies on interference in experimental influenza. I. Biological observations. *Ark. Kemi Min. Geol.*, **24B,** No. 7.

MELNICK, J. L. (1951). Latent survival of viruses and persistence of immunity. *Proc.* II *Internat. Poliomyelitis Conf.* Copenhagen.

RUSTIGIAN, R. & PAPPENHEIMER, A. M. (1949). Myositis in mice following intramuscular injection of viruses of the mouse encephalomyelitis group and of certain other neurotropic viruses. *J. Exp. Med.*, **79,** 69.

SABIN, A. B. (1941). Constitutional barriers to involvement of the nervous system by certain viruses, with special reference to the role of nutrition. *J. Pediat.*, **19,** 596.

SANDERS, F. K. (1950). Unpublished.

SANDERS, F. K. (1952). Neuronal transport of murine encephalomyelitis virus. *Proc. Roy. Soc. Lond.* B. (in press).

SANDERS, F. K. & MACDONALD, F. M. E. (1952). The development of age resistance to murine encephalomyelitis virus. *Proc. Roy. Soc. Lond.* B. (in press).

SANDERS, F. K. & STRAHAN, R. (1952). In preparation.

SCHLESINGER, R. W. (1950). Incomplete growth cycle of influenza virus in mouse brain. *Proc. Soc. Exp. Biol. Med. N.Y.*, **71**, 541.

SCOTT, T. F. M., BLANK, H., CORIELL, L. L. & CROUSE, H. (1950). Pathology and pathogenesis of the cutaneous lesions of variola, vaccinia, herpes simplex, herpes zoster, and varicella, in *The Pathogenesis and Pathology of Viral Diseases*. 1st edition. Ed. J. G. Kidd. New York. Columbia University Press.

SMADEL, J. E. & HOAGLAND, C. L. (1942). Elementary bodies of vaccinia. *Bact. Rev.*, **6**, 79.

SMADEL, J. E. & WALL, M. J. (1940). A soluble antigen of lymphocytic choriomeningitis. III. Independence of anti-soluble substance antibodies and neutralizing antibodies, and the role of soluble antigen and inactive virus in immunity to infection. *J. Exp. Med.*, **72**, 389.

SMITH, K. F. & WYCKOFF, R. W. G. (1950). Structure within polyhedra associated with insect virus diseases. *Nature*, **166**, 861.

THEILER, M. (1937). Spontaneous encephalomyelitis of mice, a new virus disease. *J. Exp. Med.*, **65**, 705.

THEILER, M. & GARD, S. (1940). Encephalomyelitis of mice: characteristics and pathogenesis of virus. *J. Exp. Med.*, **72**, 49.

WARREN, J., SMADEL, J. E. & RUSS, S. B. (1949). The family relationship of Encephalomyocarditis, Columbia SK, MM, and Mengo encephalomyelitis viruses. *J. Immunol.*, **62**, 387.

YOUNG, J. Z. (1944). Surface tension and the degeneration of nerve fibres. *Nature*, **154**, 521.

DISCUSSION

F. C. BAWDEN: Although virus particles may well undergo far-reaching changes when they enter susceptible cells, too much importance should not be given to the fact that they cannot be detected during the so-called 'dark period'. The technical difficulties of demonstrating the initial infecting particles in the presence of many host cell components, some of which may combine with the virus particles and act as inhibitors of infectivity, are great. It is rather like looking for a needle in a haystack without the help of a magnet. When tobacco leaves are inoculated with tobacco mosaic virus at different concentrations, the period during which there is no detectable increase in virus depends on the concentration of the inoculum, being shorter with more concentrated inoculum. Virus from dilute inoculum cannot reasonably be assumed to take longer to multiply and the differences will be determined by differences in the number of sites at which multiplication has occurred. Hence, in leaves infected with dilute inocula, newly-formed particles must be present at the same time as in leaves infected with more concentrated inoculum, but the greater ratio of normal plant components to virus particles in extracts of such leaves prevents the identification of the virus particles.

F. K. SANDERS: While admitting the general truth of Mr Bawden's statement, I should like to point out that in the case of GD VII virus it is likely that we *are* in fact able to demonstrate the initial infecting particles within the cells before the onset of the 'dark' period. GD VII virus presumably enters nerve cells at the periphery, and the first wave of infectivity in the hypoglossal nucleus (see Fig. 1 in paper) presumably represents virus which is already within the cells; only then does the virus disappear. Whether the disappearance occurs by combination of virus with some cell component, or by the loss of some part responsible for its infectivity, is surely irrelevant. What is important is that the 'alteration' of the virus particle does not take place until it reaches that part of the cell in which it is able to undergo multiplication.

W. M. HENDERSON: In the opening remarks with which Dr Sanders introduced his paper he stressed some of the difficulties of studying the multiplication of animal viruses, the greatest of which is probably that of isolating the virus-cell system. Although much can be done with certain viruses by using the liquid and the lining membrane of the allantoic sac of the chick embryo, this relatively simple system compared with that in the infected animal is far from ideal. *In vitro* propagation of virus in isolated susceptible tissue appears to offer many advantages and the following results have been obtained using this technique with the virus of foot-and-mouth disease. Since Maitland & Maitland (IV. *Rep. Foot and Mouth Dis. Comm.*, 1931, London, H.M. Stationery Office) and Hecke (*Zbl. Bakt.* I. Orig. 1930, **116,** 386; 1931, **119,** 385) reported the successful culture of this virus in guinea-pig embryo tissue a number of methods of *in vitro* propagation of the virus have been described but the most practicable and the one used with but slight modifications in the present experiments is that of Frenkel (*Bull. Off. Internat. Epiz.* 1947, **28,** 155).

The tissue used is the susceptible tissue from the natural host, namely, the epithelium of the bovine tongue. The scheme used is shown in fig. 6.

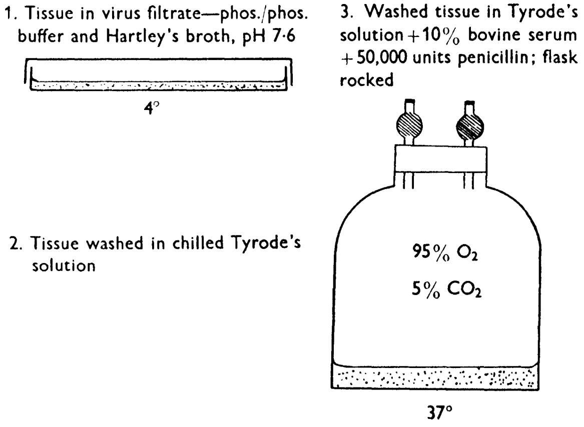
1. Tissue in virus filtrate—phos./phos. buffer and Hartley's broth, pH 7·6
4°
2. Tissue washed in chilled Tyrode's solution
3. Washed tissue in Tyrode's solution + 10% bovine serum + 50,000 units penicillin; flask rocked
95% O₂
5% CO₂
37°

Fig. 6

Minced tongue epithelium is added to a filtrate of a suspension of tissue of the previous culture passage and left for a varying period at 4°. The tissue is then removed from the filtrate and washed by repeated centrifugation in Tyrode's solution to remove the filtrate bathing the tissue. The washed tissue is added to the culture medium consisting of Tyrode's solution +10% normal bovine serum+Penicillin, the air in the flask is replaced by 95% oxygen and 5% carbon dioxide, the flask is sealed and it is rocked during the period of incubation at 37°.

When minced cattle tongue tissue is added to a filtrate of foot-and-mouth disease virus, the tissue acquires a virus content. The amount acquired is dependent upon the concentration of virus available (see Table 1). It does not appear to be dependent on the period of contact and similar results have been obtained at 4° and at 37°. I have avoided using the term 'adsorption' as adsorption and elution does not appear to occur to the same extent or in the same way as, for example, with influenza virus and susceptible tissue.

Table 1. *Acquisition of virus by tissue from suspensions of virus*

Period of Contact (Hours)	Virus Concentration (ID_{50} per g.) Filtrate	Tissue	Percentage acquired by Tissue
¾—6¾	$10^{6.15}$	$10^{4.08}$	0·9
	(Mean of 6 Observations)		
1½	$10^{5.78}$	$10^{3.9}$	1·3
1½	$10^{4.78}$	$10^{3.4}$	2·4
1½	$10^{3.7}$	$10^{2.7}$	10·0
1½	$10^{2.78}$	$10^{2.1}$	21·0

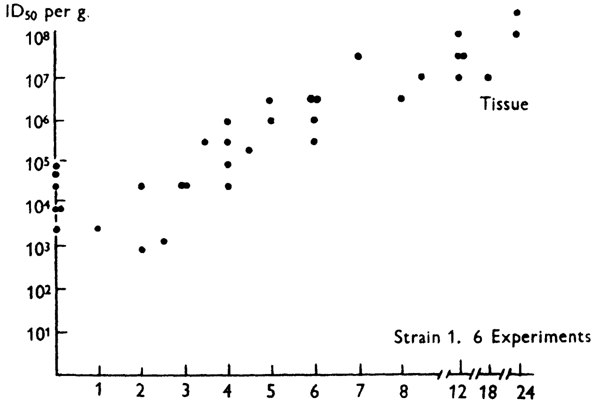

Fig. 7

Fig. 7 shows the results of determining the virus content of tissue in six experiments with one strain of virus, a parallel culture or pool of cultures being used for each determination. After an initial drop in infectivity, an increase in the virus content of the tissue is detected by the third hour. This increase proceeds during the next two hours by which time it is about 100 times the original value. After this point the rate of increase is much slower and the peak is reached between 12 and 24 hours when it is about 10,000 times the original virus content of the tissue.

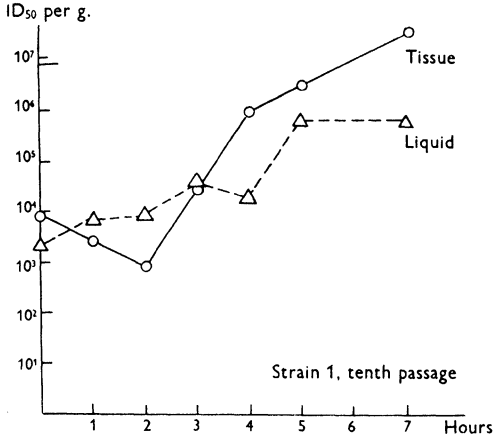

Fig. 8

Fig. 8 shows the result of one of these experiments in which the virus content of the culture liquid was also determined. This is expressed as the concentration of virus in ID_{50} corresponding to each gram of tissue in the liquid. The result suggests that the virus content of the liquid increases sharply about two hours after the first increase in the tissue which coincides with the end of the most rapid rise in the tissue.

One important aspect of the multiplication of influenza virus that has not, so far, been mentioned in this symposium is the demonstration by Henle, Henle & Rosenberg (*J. Exp. Med.*, 1947, **86,** 423) of 'one-step' growth curves. The possibility of being able to demonstrate such 'steps' in the multiplication of foot-and-mouth disease virus seemed worth investigating. If the virus acquired by the tissue, before the tissue is added to the culture liquid, had become specifically adsorbed or had become intracellular, the use of immune serum in the culture liquid instead of normal serum might reveal the first 'step' as virus released from the tissue into the liquid would be neutralized.

Fig. 9 shows the result of an experiment using immune serum in which this expectation was not confirmed. The parallel slopes of the growth curves in normal and in immune serum are similar to those found if concentrations of virus, initially made available to the tissue, differ by 100-fold. Thus, only about 1% of the initial virus content of the tissue was protected from the neutralizing action of the immune serum. This might, indeed, be all the virus actually *adsorbed.* The slight drop in

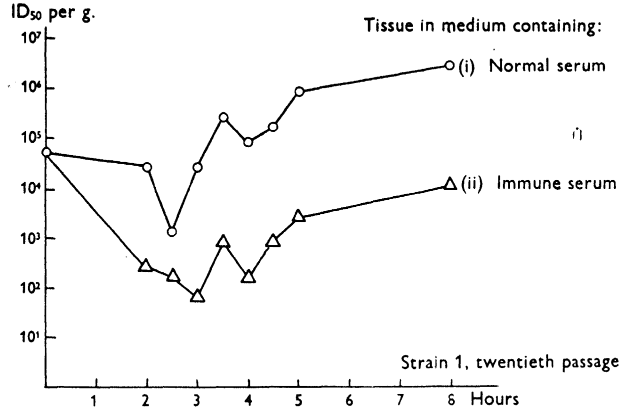

Fig. 9

infectivity of the tissue observed in both culture series at 3½ to 4 hours is consistent with the indication that the end of the first cycle of multiplication in the tissue occurs at about this point.

F. K. SANDERS: While the ideal system for studying the details of the multiplication of animal viruses is undoubtedly some form of tissue culture, the use of such methods is unlikely to yield results comparable with those achieved by workers on bacteriophage, until two conditions of culture have been fulfilled:

(1) The use of suspensions or sheets of susceptible cells in which the number of cells exposed simultaneously to a given amount of virus can be accurately determined.

(2) The use of a defined medium for the maintenance of these cells.

J. W. CZEKALOWSKI: Bauer (*Brit. J. Exp. Path.*, 1947, **28,** 440) pointed out that mouse-brains infected with some viruses (Yellow fever, Lymphocytic choriomeningitis, Lymphogranuloma) show increased dehydrogenase activity, due in part to an increase in the activity of xanthine oxidase and pyruvic oxidase. The same author found also that both chorioallantois and the chick-embryo infected with Yellow fever virus show increased xanthine oxidase activity, which is due to an increase in total xanthine oxidase of these tissues and not to the mobilization of this enzyme from the rest of the egg. The significance of the increase in xanthine oxidase is obscure since this enzyme is specific for the reactions: hypoxanthine ⟶ xanthine ⟶ uric acid (apart from its activity as the Schardinger enzyme of milk), but pyruvic oxidase may play an important part in building up the reserve of adenosine triphosphate, an important source of energy. Has Dr Sanders any comments to make on the activity of xanthine oxidase and pyruvic oxidase in nervous tissue of mice infected during his experiments with mouse encephalo-myocarditis, mouse encephalomyelitis and poliomyelitis virus?

F. K. SANDERS: We have as yet made no determinations of the enzymes you mention in nervous tissue infected by our methods.